WILD-TYPE AND MUTATED LACCASES FOR A GREEN INDUSTRY

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"Make things as simple as possible, but not simpler"

Albert Einstein

Alla mia famiglia

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Summary

In the frame of white biotechnology, enzymes attract an enormous attention both for their potential and for the recent social and economical interest in green chemistry. Thanks to the improved knowledge of production biochemistry, fermentation processes, and recovery methods, an increasing number of enzymes can be affordably produced. The majority of currently used industrial enzymes are the hydrolytic ones, being used for the degradation of various natural substances. Also, various oxidative enzymes, primarily laccases are used in industries such as the starch, textile, detergent and baking industries, and they represent a second important group of enzymes.
This project has been focused on the recombinant expression of the industrially attractive laccase POXA1b from *Pleurotus ostreatus* in *Pichia pastoris* and on exploiting its industrial application in order to substitute some chemical industrial processes with a better eco-friendly ones. An economical analysis of the recombinant POXA1b laccase production process, in terms of productivity and cost has been evaluated. Furthermore, computational analysis and site-specific mutagenesis of the POXA1b were performed to increase the enzymatic performances towards selected substrates. A POXA1b laccase immobilization process was optimized by statistic methods (Response Surface Methodology) in order to increment the operational stability of the enzyme in specific processes. Applications of the recombinant enzyme were then developed: *(i)* reduction of the phenol contents inside the fruit juices by immobilized laccase POXA1b; *(ii)* synthesis of a new eco-friendly dyes for the staining of different textile materials (nylon, cotton and wool) and *(iii)* synthesis of a new eco-friendly dye for proteins visualization on the polyacrilamide gels; *(iv)* cotton fiber functionalization with an anti-adhesive and anti-oxidant dye polymer.

Riassunto

Al giorno d'oggi l'utilizzo massivo di beni primari unito ad un aumento della produzione chimico-farmaceutica, ha portato ad un drastico incremento di rifiuti con conseguenti ripercussioni sul già fragile equilibrio ambientale. Fortunatamente, diverse industrie nel mondo sono alla ricerca di metodi alternativi di produzione, che portino a un minore impatto ambientale sia in termini quantitativi che qualitativi.
L'utilizzo inoltre di materie di scarto unito all' utilizzo di sistemi biologici per la produzione industriale, potrebbe portare sia alla riduzione dei costi di fabbricazione nonché a una più facile smaltimento di rifiuti, con benefici significativi per l'ambiente circostante e l'economia. In tale ambito si posizionano le *"white biotechnology"* o biotecnologie industriali.
Quest'ultime si propongono di sostituire processi chimici di sintesi con processi a basso impatto ambientale basati sull'uso di biosistemi - intesi sia come (micro)organismi ed enzimi - per una "Chimica verde". L'impiego degli enzimi a livello industriale è in netto aumento, come rivelato dall'espansione del loro mercato, stimato intorno ai 4 milioni di dollari nel 2013, e con una crescita annuale prevista dell' 8,3% tra il 2014 e il 2020. I principali settori industriali nei quali vengono utilizzati maggiormente gli enzimi sono alimentare, chimico, farmaceutico, tessile etc. In tali settori gli enzimi utilizzati generalmente si possono suddividere in due categorie, gli enzimi idrolitici ed quelli ossidativi.
Tra gli enzimi ossidativi le laccasi sono sicuramente quelle che mostrano una migliore applicabilità in ambito industriale. Le laccasi sono enzimi ubiquitari ad attività fenolo-ossidasica, che svolgono diverse funzioni biologiche. Grazie alla loro capacità di ossidare un'ampia gamma di substrati aromatici questi enzimi trovano applicazione in diversi settori industriali, che vanno da quello della produzione della carta, a quello tessile, alimentare, farmaceutico, cosmetico ed altri. La maggior parte delle laccasi attualmente studiate e integrate in flussi di processi industriali sono di origine fungina soprattutto grazie alla peculiare stabilità mostrata da questa classe di enzimi.
Nel presente lavoro di tesi è riportato il contributo dato ad un progetto industriale, svolto in collaborazione con due gruppi industriali (BioPox Srl e Biogrammatics Ltd), finalizzato all'ottimizzazione dei processi di produzione eterologa della laccasi di *Pleurotus ostreatus* POXA1b.
Sono poi riportati i risultati ottenuti per il miglioramento, tramite tecniche di mutazione sito-specifica, della stessa laccasi al fine di poter applicare tale enzima in alcuni settori industriali, in sostituzione dei tradizionali processi chimici.
Le applicazioni sviluppate sono poi state:

- Chiarificazione dei succhi di frutta attraverso l'impiego di laccasi immobilizzate su sopporti epossidici
- Sintesi di coloranti per la colorazione dei più comuni tessuti (cotone, nylon e lana) e per la visualizzazione di proteine su gel di acrilamide.
- Funzionalizzazione, tramite POXA1b, delle fibre di cotone al fine di prevenire l'adesione batterica e incrementarne le capacità anti-ossidanti

1) **Produzione ricombinante della laccasi POXA1b di *Pleurotus ostreatus* in *Pichia pastoris* confrontando l'efficienza di due diversi promotori, inducibile e costitutivo**

Negli ultimi anni la laccasi fungina POXA1b è stata oggetto di numerosi studi, per le sue peculiari caratteristiche che la rendono interessante da un punto di vista industriale. Tali caratteristiche sono:

- elevato potenziale redox
- stabilità a pH alcalini
- stabilità ad alte temperature

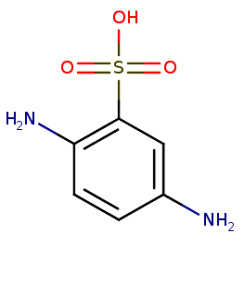
In questo lavoro di tesi è riportato il contributo dato ad un progetto volto alla ottimizzazione ed espressione ricombinante e produzione della laccasi POXA1b e successivamente è alla valutazione dell'impatto economico della sua produzione a livello industriale.

Risultati

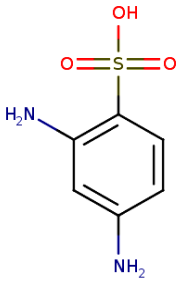
Il *cDNA* codificante per la laccasi POXA1b è stato clonato in *P. pastoris*, in due diversi vettori d'espressione. Tali vettori sono sotto il controllo di due differenti promotori, uno inducibile da metanolo (pAOX) e l'altro costitutivo (pGAP). L'espressione della proteina ricombinante in entrambi i sistemi è stata analizzata in diverse scale di produzione ed inoltre è stata ottimizzata la formulazione del terreno di coltura. I due sistemi sono risultati essere paragonabili in termini di produttività e per tanto entrambi sono stati utilizzati per valutarne il costo di produzione. Sulla base dell'analisi tecnico-economica il sistema regolato dal promotore costitutivo è risultato essere più conveniente da un punto di vista economico e soprattutto competitivo con i sistemi più utilizzati per la produzione delle laccasi ricombinanti in commercio.

2) Mutazione sito-specifica attraverso analisi computazionali della laccasi POXA1b di *Pleurotus ostreatus*

Il polimero generato dall'ossidazione del resorcinolo e 2,5-babsa da parte delle laccasi ha trovato largo impiego nel settore dei coloranti. Al fine di ridurre i costi del processo di sintesi si è pensato di sostituire il 2,5-dabsa con il suo isomero più economico, il 2,4-dabsa. Purtroppo la laccasi POXA1b non è in grado di ossidare tale composto. Pertanto studi computazionali sono stati necessari al fine di *(i)* identificare le ragioni molecolari della mancata reattività dell'enzima verso il substrato e *(ii)* elaborare mutazioni sito specifiche che possano portare ad un aumento della capacità di riconoscimento da parte di POXA1b del 2,4-dabsa e quindi catalizzarne l'ossidazione



2,5-dabsa



2,4-dabsa

Risultati
Le analisi computazionali effettuate hanno condotto all' identificazione e la sostituzione di tre amminoacidi responsabili sia dell' accessibilità del 2,4-dabsa al sito attivo e sia della reazione d' ossidazione.
In seguito i *cDNA*, recanti le mutazione individuate, sono stati espressi e prodotti in via eterologa in *P. pastoris* e la loro attività è stata saggiata verso la molecola *target.* I mutanti selezionati non hanno però portato alcun miglioramento nella reazione d'ossidazione del substrato rispetto alla proteina selavatica. Si è ipotizzato che tale mancanza d'attività non fosse dovuta al non corretto posizionamento del substrato nella sito attivo dell' enzima, ma bensì all'elevato potenziale *redox* necessario all'ossidazione della molecola stessa. Nonostante le varianti di POXA1b non siano attive verso il 2,4-dabsa, la loro tendenza di ancorare il gruppo carico negativamente nel sito attivo potrebbe però facilitare l'ossidazione di altre molecole strutturalmente simili. Sono state quindi selezionate tre moleocle rappresentanti di due differenti classi di sostanze, acidi idrossicinnamici e triidrossibenzoici, che oltre ad possedere analogie strutturali con il 2,4-dabsa trovano anche utilizzo in diversi settori industriali. I mutanti di POXA1b sono stati purificati e si è verificata la loro attività verso le molecole identificate. I test hanno dimostrato un aumento significativo sia in termini di affinità che di efficienza catalitica dei mutanti rispetto alla proteina non mutata.

3) Immobilizzazione della laccasi POXA1b e suo utilizzo nella chiarificazione dei succhi di frutta

Il non ottimale utilizzo degli enzimi in ambito industriale è in genere dovuto alla loro bassa stabilità e all'alto costo di produzione. Per questi motivi, negli ultimi anni, la ricerca è stata improntata allo sviluppo di sistemi enzimatici immobilizzati su differenti supporti. Tali processi migliorano in genere la stabilità dell'enzima, in quanto risulta essere meno soggetto agli stress meccanici ed inoltre permettono il suo riutilizzo all'interno del processo produttivo, riducendone sensibilmente i costi.
In questo lavoro è stato ottimizzato il sistema di immobilizzazione della laccasi POXA1b, al fine di rendere possibile la sua applicazione nella chiarificazione dei succhi di frutta. Con il termine "chiarificazione" ci si riferisce alla riduzione di fenoli all'interno delle bevande a base di frutta, che sono spesso causa di sapore sgradevoli e imbrunimento del prodotto.

Risultati
In questo lavoro di tesi la laccasi POXA1b è stata immobilizzata attraverso legame covalente a supporti di natura epossidica. L'ottimizzazione del processo di immobilizzazione è stato sviluppato tramite l'utilizzo di un metodo statistico, quale *Response Surface Methodology* (RSM). Tale analisi ha permesso di individuare quali possano essere i fattori chiave nell'ottimizzazione del processo d' immobilizzazione.
Al termine di questo studio, i parametri identificati hanno portato ad una resa di immobilizzazione pari al 98%. L'enzima immobilizzato è stato inoltre caratterizzato e messo al confronto con quello libero per valutare le variazioni delle prestazioni enzimatiche. Da tale confronto è stato verificato che il processo d'immobilizzazione ha aumentato la stabilità dell'enzima in un ampio intervallo di temperature e pH, facilitandone quindi l'utilizzo in ambito industriale.
In seguito POXA1b immobilizzato è stato utilizzato per mettere a punto un processo di trattamento di succhi di frutta al fine di abbatterne il contenuto in fenoli. Il processo

sviluppato ha ridotto sensibilmente la presenza dei fenoli nelle bevande analizzate, dimostrando in questo modo l'applicabilità industriale del sistema stesso.

4) Sintesi catalizzata da laccasi di coloranti eco-sostenibili

La maggior parte dei coloranti utilizzati sono molecole di sintesi chimica. Grazie alle biotecnologie, si sta tentando di sostituire alcune fasi della sintesi con l'uso di biocatalizzatori riducendo quindi l'impiego di condizioni operative estreme ed, spesso, riducendo i tempi ed i costi del processo di sintesi, riuscendo in alcuni casi, anche ad abbattere i costi connessi con 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 ed altri composti aromatici con la formazione di polimeri colorati. Attualmente, diverse laccasi di origine fungina sono già state utilizzate per tale produzione. Nel presente studio la laccasi POXA1b è stata utilizzata per la sintesi di due nuove molecole coloranti con diverse possibilità applicative.

Risultati
Attraverso l'utilizzo della laccasi POXA1b è stato possibile sintetizzare e ottimizzare la produzione di due diversi coloranti. Il primo colorante è stato sintetizzato a partire da due differenti precursori quali, resorcinolo e 2,5-dabsa (BROWN_1), mentre per il secondo colorante sono stati utilizzati α-naftolo e 2,5-dabsa (SIC_RED). I due coloranti sono stati successivamente impiegati in due processi distinti:

Colorazione dei tessuti
BROWN_1 è stato utilizzato per la colorazione dei più comuni tessuti impiegati nell'industria tessile: nylon, cotone e lana. I tessuti colorati sono stati soggetti a test per stabilirne la qualità della colorazione. I risultati ottenuti si sono dimostrati essere conformi ai standard richiesti.

Visualizzazione di proteine su gel d' acrilammide
SIC_RED è stato utilizzato per la visualizzazione di proteine su gel d' acrilamminde confrontandolo con il principale colorante presente in commercio, il Blue di coomassie. Dal confronto dei due sistemi di colorazione, il colorante neo-sintetizzato è risultato essere sia in termini di specificità di substrato, sensibilità, tempi e costi migliore di quello commerciale.

5) Funzionalizzazione delle fibre di cotone catalizzata da laccasi, al fine di conferirne proprietà anti-adesive e anti-ossidanti

Negli ultimi decenni il consumo *pro capita* di fibre tessili, sintetiche e naturali, è aumentato più del 100% ed è in continua espansione. In particolare, il cotone ricopre gran parte del mercato delle fibre naturali, trovando impiego in diversi settori dell'industria, da quello tessile a quello medicale. Questa fibra, però presenta diversi svantaggi specialmente nella sua colorazione, in quanto i principali coloranti ionici utilizzati riescono a legare la fibra solo ad alte concentrazioni saline e con l aggiunta di additivi chimici. Queste sostanze sono spesso tossiche e di difficile smaltimento, rendendo quindi il processo poco eco-sostenibile e dispendioso da un punto di vista economico. Inoltre, il cotone essendo una molecola di origine naturale, è soggetta a

colonizzazioni da parte di microorganismi, spesso patogeni. In quest' ottica tale lavoro di tesi si è incentrato nello sviluppo di una molecola che sia in grado di colorare in modo efficiente il cotone e al tempo stesso prevenire l'adesione cellulare.

Risultati

Il colorante, la cui sintesi è descritta nel capitolo precedente, sintetizzato a partire da resorcinolo e 2,5-dabsa è stato utilizzato per la funzionalizzazione delle fibre di cotone. Tale processo è stato sviluppato in modo tale che la laccasi, in un unico passaggio, polimerizzi i precursori fenolici e contestualmente favorisca il loro legame alla superficie del tessuto. Il prodotto così ottenuto è stato sottoposto a test per valutarne le proprietà anti-ossidanti e anti-adesive. I dati ottenuti hanno confermato che la funzionalizzazione è in grado di colorare il tessuto e che il prodotto generato possiede elevate capacità anti-ossidanti e previene in maniera efficace l'adesione cellulare dei microorganismi.

Conclusioni

In conclusione il seguente progetto di dottorato ha portato:

1) All'espressione e produzione della laccasi fungina POXA1b.

2) Allo sviluppo di forme mutate della laccasi POXA1b attive verso nuovi substrati.

3) Allo sviluppo di un efficiente metodo di immobilizzazione della laccasi POXA1b

4) Allo sviluppo di nuovi processi industriali ecosostenibili quali:

- chiarificazione di succhi di frutta;
- sintesi di due coloranti innovativi;
- preparazione di fibre di cotone con proprietà anti-adesive e anti-ossidanti.

CHAPTER 1

Introduction

1.1. Enzymes in the industry

Nowadays, the production and abuse of daily life products such as food, feed, textile, paper, chemicals and pharmaceuticals, combined to the growing global population have caused the increment of wastes with a negative impact on environment (OECD, 2009; European Commission, 2009). Different industries in the world are thus looking for alternative technologies with a lesser impact on the environment. The use of bio-based materials and nature's production processes, known as industrial biotechnology (Kirk *et al.,* 2002; Soetaert and Vandamme, 2010), is one of the alternative technology which could be used to either replace or supplement conventional technologies in moving towards cleaner production processes (Kirk *et al.,* 2002; Bornscheuer and Buchholz, 2005; OECD, 2009; Haas *et al.,* 2009; Wohlgemuth, 2009). Biotechnology offers an increasing potential for the production of goods that satisfy 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 both for their potential and for the recent social and economic interest in green chemistry. In 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 affordably produced. Moreover, advances in the application of enzymes have greatly expanded their 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 s2013, 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). 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 application field of industrial enzyme due to versatility of their applications as well as to the stringent government rules about the utilization of synthetic food ingredients. The majority of currently used industrial enzymes are the hydrolytic ones, used for the degradation of various natural substances (Tab. 1). Proteases remain the dominant enzyme type,

for their extensive use in the detergent and dairy industries. Different oxidative enzymes, primarily laccases, peroxygenases and peroxidases, used in industries such as the starch, textile, detergent and baking industries, represent the other important group of enzymes (Kirk *et al.,* 2002). This project was focused on the application of a new laccase in different industrial fields.

Table 1 Enzymes used in various industrial segments and their applications (Kirk *et al.,* 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

1.2 Laccases

1.2.1 General features

Laccases (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) are multicopper oxidases catalyzing the oxidation of a wide range of phenolic substrates. These enzymes 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 in bacteria (Octavio et al., 2006). These class of enzymes have various biological functions depending on the different micro-organism source, such as degradation of complex polymers (lignin, humic acid), lignification, detoxification, pathogenicity, morphogenesis; sporulation is characteristic of fungal laccases, while copper detoxification and spore coat resistance is usual for the bacterial ones. (Strong and Claus, 2011).

Among laccases, the fungal ones are the most studied and characterized enzymes, and have been currently used in many biotechnological applications (Pezzella *et al.,* 2015).

1.2.2 Structure and Mechanism of fungal 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).

These enzymes contain four copper atoms: one paramagnetic type 1 copper (T1 Cu) that is responsible for their characteristic blue colour and where the oxidation of the reducing substrate occurs, one type 2 copper (T2 Cu) and two type 3 coppers (T3 Cu) that form a trinuclear cluster in which molecular oxygen is reduced to two molecules of water (Davies and Ducros, 2006; Mot and Silaghi Dumitrescu, 2012) (Fig. 1). The substrate range of laccases is very broad as they can oxidize aromatic compounds (ortho- and para-diphenols, methoxysubstituted phenols, diamines, benzenethiols), metal ions (Mn^{2+}), organometallic compounds (e.g. $[W(CN)_8]^{4-}$ -, $[Fe(EDTA)]^{2+}$), organic redox compounds (e.g. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), ABTS; 1- hydroxybenzotriazole, HBT) and the iodide anion (Morozova *et al.,* 2007; Xu, 1996). Furthermore, in the presence of both natural and synthetic redox mediators, the catalytic activity of these enzymes may be expanded to non-phenolic substrates that are very recalcitrant and hardly oxidized by laccase alone (e.g. polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls, azo-dyes or organophosphate pesticides) (Cañas and Camarero, 2010). From an electrochemical viewpoint and based on the analysis of their molecular structures, these enzymes are classified into three different groups according to the redox potential of the T1 site ($E^{0'}{}_{T1}$): low-, medium-, and high-redox potential laccases (Mot and Silaghi Dumitrescu, 2012). The $E^{0'}{}_{T1}$ of laccases is not determined by a single structural feature but it is rather the result of the combination of various factors, such as copper–ligand interactions, the effects of desolvation around the T1 site, the intermolecular electrostatic interactions, and the restrictions in protein folding (Li *et al.,* 2004). Bacterial and plant laccases constitute the group of low-redox potential laccases, with an $E^{0'}{}_{T1}$ below +460 mV vs. NHE (Normal Hydrogen Electrode) and a Met residue as the T1 Cu axial ligand. Fungal laccases fall into the medium- and high-redox potential classes. The group of medium redox potential laccases includes enzymes from ascomycetes and basidiomycetes fungi, with an $E^{0'}{}_{T1}$ ranging from +460 to +710 mV vs. NHE, and typically with a Leu as the non-coordinating axial

ligand. High redox potential laccases (HRPL) are mainly produced by basidiomycete white-rot fungi, with an $E^{0'}_{T1}$ ranging from +730 to +790 mV vs. NHE, and with a Phe as the non-coordinating axial ligand. This latter group is the most relevant for distinct industrial applications, since their $E^{0'}_{T1}$ allows HRPL to oxidize a much wider range of substrates than their low- and medium-redox potential counterparts (Rodgers *et al.,* 2010).

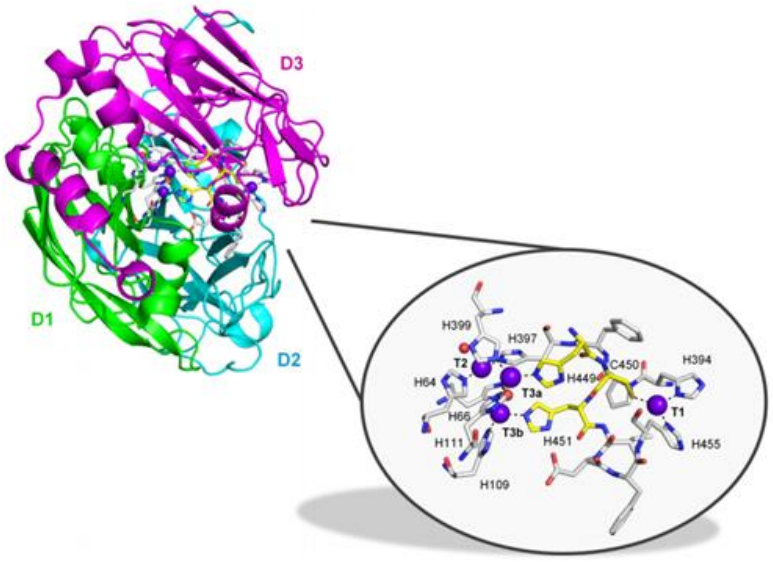


Fig. 1. General structure and details of the active site of laccase (*Trametes trogii* laccase, PDB ID: 2HRG). Purple blue spheres represent copper ions and red spheres depict coordinating water molecules. The residues of the internal transfer pathway from T1 Cu to the T2/ T3 trinuclear cluster are colored in yellow. Residues involved in the first coordination sphere of the catalytic coppers and their interactions (as black dashes) are also represented.

1.2.3 Industrial applications of laccases

First uses of laccases in industry date back to 1990s (Giardina *et al.,* 2010, Piscitelli *et al.,* 2013). 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 reaching a peak in the last 10 years (Pezzella *et al.,* 2015).

The most prominent industrial fields include:

- Food industry: in beverage processing (wine, fruit juice and beer), in the determination of ascorbic acid and in the gelation of sugar beet pectin (Osma *et al.*, 2010) or in the clarification of orange juice (Lettera *et al.,* 2015)
- Paper industry: in the chlorine-free bleaching of paper pulp and in effluent treatment (Couto and Herrera, 2006).
- Textile industry: in fibre modification, fabric bleaching and in the degradation of wastewater dyes (Couto and Herrera, 2006).
- Paint industry: laccase-mediator systems for the drying of alkyd resins widely employed in paints and coatings (Greimel *et al.,* 2013).
- Organic chemistry: in the oxidative coupling of radical intermediates to obtain antitumor compounds (e.g. actinocin or vinblastine), cyclosporin derivatives (e.g. cyclosporin A), hormones (e.g. β- estradiol) and phytoalexins (e.g. resveratrol), (Kunamneni *et al.,* 2008a) as well as in the enzymatic derivatization of amino acids such as L-tryptophane, L phenylalanine or L-lysine (Mogharabi and Faramarzi, 2014).
- Biofuels: to remove phenolic compounds that inhibit the fermentation of the sugars present in the hydrolysate of lignocellulosic materials (Kudanga and Le Roes-Hill, 2014).

- Soil bioremediation: fungal laccases degradation of endocrine disrupting compounds, PHAs and dangerous phenols. (Macellaro *et al*., 2014, Wu *et al.,* 2008 and Ahn *et al.,* 2002) .
- Cosmetics industry: in the preparation of hair dyes and skin lightening preparations (Couto and Herrera, 2006).
- Biomedical industry: laccase immobilization in strips or bandages to be used for the diagnosis of fungal infections (Schneider *et al.,* 2012).
- Nanobiotechnology: in the development of biofuel cells and biosensors to detect different compounds and metabolites (Kunamneni *et al.,* 2008b).

1.2.4 Laccase engineering

Nowadays, industry sectors demand enzymes more efficient, more stable at hard reaction conditions and with reduced production costs. A promising solution for these request is enzyme engineering (Schafer *et al.,* 2007). In fact, industrial laccases often require additional engineering to increase stability and activity, and to turn off/on specificity towards target substrates. Generally there are two main strategies for their engineering: directed evolution and rational design, which can be combined to semi-rational design or focused directed (designed) evolution (Fig.2) (Steiner *et al.,* 2012). Directed evolution can be achieved by two major approaches, either by randomly recombining a set of related sequences (e.g. gene shuffling), or by introducing random changes in single protein sequences (e.g. error-prone PCR). The advantage of directed evolution is that no structural information is needed and that variations at unexpected positions distant from the active site can be introduced. However, the changes are usually small and several rounds of evolution have to be applied and thus a high number of variants have to be screened, which is time and labor consuming and requires cheap, fast and reliable high-throughput assays.

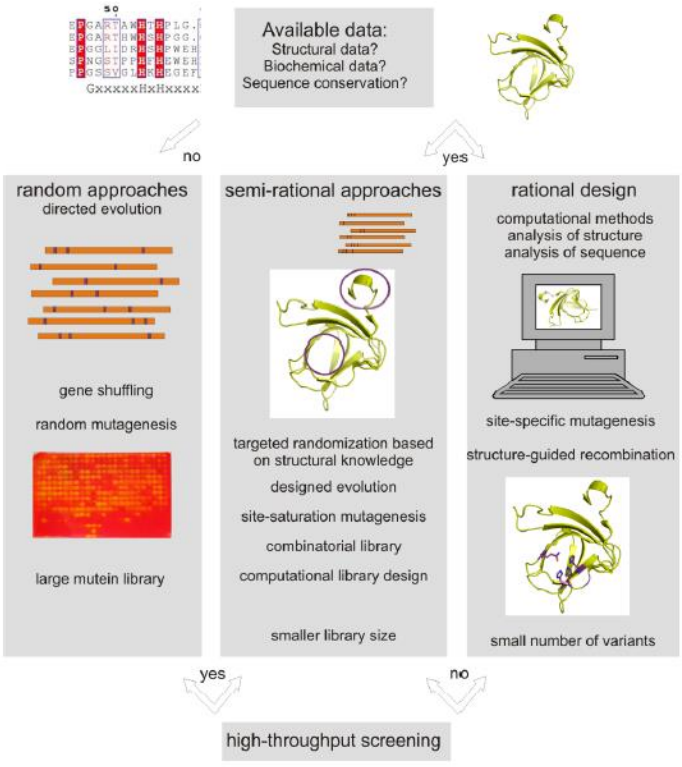


Fig. 2. Overview of approaches for protein engineering by random, rational and combined methods (Steiner *et al.,* 2012)

With the availability of an increasing number of protein structures or reliable models, biochemical data and computational methods, enzyme engineering is developing more and more from random approaches (directed evolution) to semi-rational or rational (data-driven) design. In rational design biochemical data, protein structures and molecular modeling data are evaluated to propose mutations, which are introduced by site-specific mutagenesis. One of the advantages of a rational design approach is an increased probability of beneficial mutations and a significant reduction of the library size and thus less effort and time has to be applied for the screening of the library. This is especially advantageous if no high-throughput assay system is available. Nonetheless this approach requires mechanistic information, computational studies based on bioinformatics (Yang *et al.,* 2012), molecular modeling (quantum and classical simulations),(Privett *et al.,* 2012) or *de novo* design (Eiben *et al.,* 2012) (including combinations with directed evolution (Khersonsky *et al.,* 2012)). This information, however, has been typically limited to the active site. In these regards, rational engineering should aim for a complete description of the enzymatic process: (1) substrate migration and recognition, typically studied by biophysical techniques; and (2) the biochemical reaction, modeled by quantum chemistry methods.

Semi-rational methods combines advantages of rational and random protein design, creating smaller and smarter libraries of mutants based on knowledge derived from biochemical and/or structural data (Chica *et al.,* 2005). An example for a semi-rational approach is CASTing (combinatorial active site saturation test), which uses the information derived from e.g. structural data to identify amino acids in interesting regions (e.g. active site), which are then mutated randomly or by site-saturation mutagenesis one by one or in combination (Abrahamson *et al.,* 2012, Dudek *et al.,* 2011, Reetz *et al.,* 2006). Random combination of mutations or correlated mutations at targeted positions can result in synergistic effects that might have been missed in single site-specific mutagenesis. However, these combinatorial approaches increase the library sizes terribly and various computational methods have been developed in recent years, that help to decrease the library size by screening of virtual libraries and eliminating mutations predicted to be unfavorable for the protein fold (Chen *et al.,* 2012, Lappe *et al.,* 2009, Lippow *et al.,* 2010, Treynor *et al.*, 2007).

1.2.4 Laccases from Pleurotus ostreatus

The white-rot fungus *P. ostreatus*, belonging to basidiomycetes, deserves attention for fundamental studies in mycology and for many biotechnological applications. Indeed, this fungus has been widely exploited for its abilities in bioremediation processes (Olivieri *et al.,* 2012; Giardina *et al*., 2009; Karas *et al*., 2011) and can be considered an invaluable source of oxidases. In particular, six different *P. ostreatus* laccases isoenzymes have been isolated and characterised so far (Piscitelli *et al.,* 2013). These isoenzymes represent a variegated group of laccases endowed with peculiar properties. LACC10 (POXC) is a canonical laccase, most abundantly produced under different growth conditions (Palmieri *et al*., 1993). LACC6 (POXA1b) is a neutral blue laccase very stable at alkaline pH (Macellaro *et al*., 2014) and with a high redox potential (Garzillo *et al.,* 2001), characteristics that further enlarge the range of its potential biotechnological application (Alcalde 2007). This last enzyme (POXA1b) has been successfully used as template for the selection of random mutants with further improved features (Festa *et al.,* 2008; Miele *et al.,* 2010). LACC2 (POXA3) is an atypical heterodimeric laccase, constituted by a large subunit, clearly

homologous to other fungal laccases, and a small subunit ssPOXA3, probably involved in the stabilization of the complex (Giardina *et al*., 2007; Faraco *et al*.,2008).

1.3 Aim of the thesis

Aim of this PhD project was to heterologously express, produce and engineering the attractive laccase POXA1b from *P. ostreatus* in *Pichia pastoris* in order to apply this enzyme in several industrial fields. The application performed in this project have been:

- clarification of fruit juices by immobilized laccase POXA1b
- synthesis of different "green" dyes for the staining of different materials and proteins on the polyacrilamide gels
- functionalization of cotton fiber with an anti-adhesive and anti-oxidant dye polymer.

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CHAPTER 2

Evaluation of techno-economic feasibility of recombinant laccase production in *Pichia pastoris*: a comparison between inducible and constitutive promoters

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Evaluation of techno-economic feasibility of recombinant laccase production in *Pichia pastoris*: a comparison between inducible and constitutive promoters

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ABSTRACT
The heterologous production offers viable opportunities to tailor laccase properties to specific industrial needs, however few examples of recombinant laccases are available on the market. The high redox potential laccase POXA1b was chosen as case study to be lunched on the market, due to its desirable properties in terms of activity/stability profile, and already assessed applicability. The economic feasibility of recombinant laccase production was simulated through Superpro Designer 9.0®. Two scenarios were evaluated: Scenario (I) production based on methanol inducible system, AOX; Scenario (II) production based on the constitutive system, GAP, fed with glycerol.
The cost forecast for POXA1b production is around 10^{-4} € U^{-1} and is competitive with the current laccase price.

Keywords: cost analysis; recombinant laccase; constitutive production

2.1 Introduction

Laccases (*p*-diphenol-dioxygen oxidoreductases; EC 1.10.3.2) are oxidative enzymes coupling the four single-electron oxidations of a broad spectrum of reducing substrates to the reduction of molecular oxygen (Giardina *et al.* 2010). The production of water as the only by-product makes laccases one of the ‘‘greenest’’ enzymes of the 21st century (Maté *et al.* 2010).

Despite having very similar structures, laccases exhibit a wide range of redox potential, from +0.430 to +0.800 V (*vs.* normal hydrogen electrode) (Cambria *et al.* 2012). The redox potential is a key parameter affecting substrate specificity since the higher is the laccase redox potential, the wider is the range of oxidised substrates. Fungal laccases display a higher redox potential respect to that of laccases from other sources, and find effective exploitations in several industrial applications (Pezzella *et al.* 2015).

The versatility and scaling-up possibilities of recombinant enzyme production opened up new commercial opportunities for their industrial uses. In order to meet the increasing market demand for high redox potential laccases, interest in their heterologous expression in different hosts has been raising (Piscitelli *et al.* 2010).

The fungal laccase POXA1b (Giardina *et al.* 1999; Garzillo *et al.* 2001) from *Pleurotus ostreatus* is an important representative member of high redox potential laccases. In fact, this laccase displays a well-known activity and stability at alkaline pH and high temperature, features rendering it a suitable candidate for a wide range of industrial sectors, such as chemical, pulp and paper, bioremediation. Such an attractive enzyme has encouraged a deep investigation on the optimization of its production (Piscitelli, 2005; Macellaro 2014), and on the molecular characterization of its variants (Miele *et al.* 2010; Macellaro *et al.* 2014; Giacobelli *et al.* 2017). Moreover, it has been successfully tested in different applications, such as in the bioremediation field (Miele *et al.* 2009; Macellaro *et al.* 2014), in fruit juice clarification (Lettera *et al.* 2016), and in dye synthesis (Pezzella *et al.* 2016).

POXA1b is an ideal candidate for the laccase market since it brings together several elements necessary for an effective laccase exploitation: *i)* stability and activity in a wide range of pHs and temperatures; *ii)* high redox potential; *iii)* already assessed applicability in several fields; *iv)* high production level in heterologous hosts; *v)* availability of collection of evolved variants to be tailored to specific industrial needs.

Biopox s.r.l. is a small Italian biotech company involved in the design and development of green enzyme based processes having in its portfolio, among others, different oxidative enzymes. In this article the knowledge of parameters influencing cost production of POXA1b from the heterologous host *Pichia pastoris* has been deepened. The economic analysis of the POXA1b productive process in two different expression systems is herein presented.

2.2 Materials and methods

2.2.1 Materials
Reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Expression vectors pPICZB and pJGGαKR were purchased from Invitrogen (Carlsbad, CA, USA) and Biogrammatics, Ltd (Las Palmas Dr, Carlsbad, CA, USA), respectively. DNA restriction and modifying enzymes were supplied by Promega (Madison, WI, USA). Culture media were bought from BD Difco (Becton Drive, Franklin Lakes, NJ USA).

2.2.2 Strains and plasmids
Escherichia coli strain Top 10 (F-*mcrA* D (*mrr-hsdRMS-mcrBC*) *f80lacZDM15 DlacX74 deoR recA1 araD139 D* (*ara-leu*) *7697 galU galK rpsL (StrR) endA1 nupG*) was used in all DNA manipulation. *E. coli* was grown in Luria-Bertani (LB) medium (10 g/l bacto tryptone, 10 g/l NaCl, 5 g/l yeast extract). Selective medium was supplemented with 100 µg/ml of ampicillin antibiotic. LB low salt (10 g/l bacto tryptone, 5 g/l NaCl, 5 g/l yeast extract) was required for manipulation of pPICZB and pJGGαKR plasmids in *E. coli*.
The plasmids pPICZB and pJGGαKR were used for the expression of POXA1b in *P. pastoris*. The *P. pastoris* strain used for heterologous expression was BG-10 (BioGrammatics Ltd). *P. pastoris* strain was propagated in YPDS medium (10 g/l yeast extract; 20 g/l bacto tryptone; 20 g/l glucose; 182.2 g/l sorbitol).

2.2.3 Vectors construction and transformation
A gene coding for POXA1b from *P. ostreatus* was synthesized and optimized according to *P. pastoris* codon usage (Thermo Fischer Scientific, Waltham, Massachusetts, USA). The gene was properly designed in order to allow its cloning into the expression plasmids. The gene product was: *i)* restricted with *EcoR*I-*Xba*I for cloning into pPICZB under the control of AOX1 promoter to get the recombinant plasmid pPICZB-POXA1b and *ii)* hydrolyzed with *BsaI* and ligated into the corresponding site of the pJGGαkR vector in-frame with the α-factor (signal peptide) under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGG-POXA1b. Both recombinant plasmids were linearized by *BsiWI* and transformed into *P. pastoris* BG10 by electroporation, as already reported by Piscitelli et al. (Piscitelli, 2017).
The cell suspensions were spread on YPDS medium supplemented with 100 µg/ml Zeocin or 900 µg/ml G418 respectively for pPICZB-POXA1b and pJGG-POXA1b transformations, and incubated for 3-7 days at 28°C until colony formation. The colonies were then streaked on solid MM medium (13 g/l yeast nitrogen base with ammonium sulfate without amminoacids, $4x10^{-4}$ g/l biotin, 1% methanol, 0.6 mM $CuSO_4$, 100 µg/ml Zeocin) for pPICZB-POXA1b and MD medium (13 g/l yeast nitrogen base with ammonium sulfate without amminoacids; $4x10^{-4}$ g/l biotin; 20 g/l glucose, 0.6 mM $CuSO_4$, and 900 µg ml^{-1} G418) for pJGG-POXA1b. Both media were supplemented with the laccase chromogenic substrate ABTS (0.2 mM), to verify laccase expression. Plates were incubated upside down for 4 days at 28°C and checked for the development of green colour. Positive clones were inoculated in liquid media (BMGY for pJGG-POXA1b bearing clones and BMMY for pPICZB-POXA1b ones), incubated at 28°C for 8 days on a rotary shaker (250 rpm), and daily assayed for cell density and secreted laccase activity. Media composition was as follows: 13 g/l yeast nitrogen base with ammonium sulfate without amminoacids, 10 g/l yeast extract; 20 g/l peptone, 100 mM potassium phosphate, pH 6.0; $4x10^{-4}$ g/l biotin, 1% glucose (in BMGY) or 0.5% methanol (in BMMY). The best producing clones for each transformation, namely AOX system (among the pPICZB-POXA1b bearing clones) and GAP system (among the pJGG-POXA1b recombinant clones) were selected and used for the recombinant expression.

2.2.4 Recombinant laccase production in shaken flask
GAP system: the selected recombinant clone was pre-inoculated in 50 ml BMGY medium and grown overnight .at 28° C on a rotary shaker (250 rpm). The pre-culture was then diluted into 250 ml in 1 l flask of the same medium to get a starting OD_{600} value of 1.0. 1% glycerol

or glucose were tested as alternative carbon sources in BMGY. Cells were grown for 9 days on a rotary shaker (250 rpm) at 28°C. The supernatant was daily recovered and assayed for laccase production.
AOX system: the selected recombinant clone f was pre-inoculated in 50 ml BMGY medium. This pre-culture was overnight grown at 28° C on a rotary shaker (220 rpm) and used to inoculate 1 l shaken flasks containing 250 ml of of BMMY to get a starting OD_{600} value of 1.0. Cells were grown for 9 days on a rotary shaker (250 rpm) at 28°C and culture supernatants daily recovered and assayed for laccase activity. 1.5 % methanol was daily added to the culture to induce protein expression.

2.2.5 Laccase assay
Laccase activity was assayed at 25°C by monitoring the oxidation of ABTS at 420 nm (ε_{420}=36×10^3 M^{-1} cm^{-1}). The assay mixture contained 2 mM ABTS in 0.1 M sodium citrate buffer (pH 3.0).
Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing 1 mM DMP in the McIlvaine's citrate-phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm (ε_{477}= 14.8 x 10^3 M^{-1} cm^{-1}).
K_m values were estimated using the software GraphPad Prism (GraphPad Software, La Jolla, California- USA http://www.graphpad.com) on a wide range of substrate concentrations (0.05 - 3 mM). Enzyme activity was expressed in international units (IU).

2.2.6 Recombinant laccase production in Fed batch fermentation
Fed-batch fermentations of the constitutive GAP system were performed with an Applikon mini-bioreactor with a final working volume of 2 l. The media were as follows. PTM_1 trace salts stock solution contained, per liter: 6.0 g $CuSO_4$_$5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4$_H2O, 0.2 g Na_2MoO_4_$2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4$_$7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4 (95–98%). Batch medium contained, per liter: 23.7 ml H_3PO_4 (85%), 0.6 g $CaSO_4$_$2H_2O$, 9.5 g K_2SO_4, 7.8 g $MgSO_4$_$7H_2O$, 2.6 g KOH, 40 g glycerol,0.5 ml Antifoam, 4.4 ml PTM_1 trace salts stock solution and 0.6 mM $CuSO_4$_$5H_2O$. The feed solution contained, per liter: 550 mL glycerol or 550 g of glucose and 12 ml PTM_1 trace salts stock solution. The dissolved oxygen was controlled at DO ≈30% with a stirrer speed of 600–1200 rpm. Aeration rate was 100 l x h^{-1} air. The temperatures were 28°C, and the pH was controlled with NH_3 (28%). Before starting the fermentation, the pH of the batch medium was set to 5.0 with NH_3 (28%). For the GAP system based strain, the batch phase of 24 h was followed by the glycerol feed (feed rate 7.5 ml/h*liter fermentation volume) or glucose (feed rate 7.5 ml/h*liter fermentation volume). The fermentation was terminated when the maximum volumetric capacity of the reactor was reached (4 days). Samples were taken frequently and processed as described below.
Fed-batch fermentations of the inducible AOX system were performed with an Applikon mini-bioreactor with a final working volume of 2 l. The media were as follows. PTM_1 trace salts stock solution contained, per liter: 6.0 g $CuSO_4$_$5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4$_H2O, 0.2 g Na_2MoO_4_$2H_2O$, 0.02 g H_3BO_3, 0.5 g $CoCl_2$, 20.0 g $ZnCl_2$, 65.0 g $FeSO_4$_$7H_2O$, 0.2 g biotin and 5.0 ml H_2SO_4 (95–98%). Batch medium contained, per liter: 26.7 ml H_3PO_4 (85%), 1 g $CaSO_4$_$2H_2O$, 18.2 g K_2SO_4, 7.8 g $MgSO_4$_$7H_2O$, 2.6 g KOH, 40 g glycerol,0.5 ml Antifoam, 4.4 ml PTM_1 trace salts stock solution and 0.6 mM $CuSO_4$_$5H_2O$. Methanol feed solution contained, per liter: 300 ml methanol (100%), 700 ml H_2O and 12 ml PTM_1 trace salts stock solution. The dissolved oxygen was controlled at DO ≈30% with a stirrer speed of 600–1200 rpm. Aeration rate was 100 l x h^{-1} air. The temperature was 28°C, and the pH was controlled with NH_3 (28%). Before starting the fermentation, the pH of the batch medium was set to 5.0 with NH_3 (28%). For the AOX system based strain, the batch phase of 24 h was followed by the methanol feed (feed rate 2 ml/h* liter fermentation volume). The fermentation was terminated when the maximum volumetric capacity of the reactor was reached (8 days). Samples were daily taken frequently and processed as described below.

2.2.7 rPOXA1b recovery and characterization

The recombinant proteins were recovered at the end of the growth and the cells were removed by centrifuging for 20 min at 7,000 rpm at 4 °C. The supernatant was recovered, concentrated and dialyzed towards 50 mM Tris-HCl buffer, pH 8, in a Pall multi-cassette system (10 KDa cutoff membrane) (Pall Corporation, New York, USA). The protein concentration was determined using the Bradford method (BioRad) according to the manufacturer's instructions and using BSA as the standard.

2.2.8 Techno-economic analysis

Cost analysis of laccase production was performed by implementing the process (fermentation, biomass separation and enzyme concentration) in Superpro Designer 9.0 (Intelligen Inc.). Mass and energy balances have been performed to evaluate electricity consumption and utilities duties at each unit operation. Figure 1 reports the flowsheet of the process, consisting of a pre-culture carried in shaken flasks, fermentation, harvesting by centrifugation and concentration by ultrafiltration.

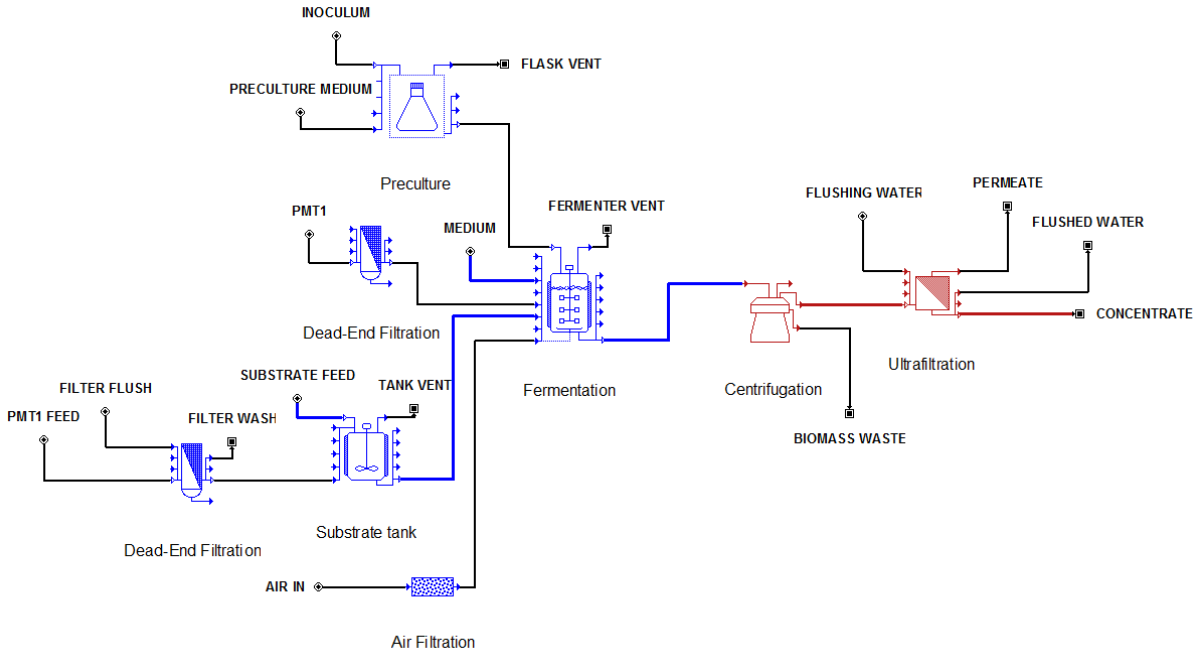


Figure 1. Flowsheet of fermentation and downstream process for Laccase production from *P. pastoris*

The chosen scenario describes the case of BIOPOX s.r.l.: a small-medium enterprise, hosted by the Department of Chemical Sciences of the Università degli Studi di Napoli Federico II (Italy) and sharing big equipments with it. According to that, Italy was chosen as location for fixing the cost of labor, electricity and other utilities as reported in Table S1.

Capital (CAPEX) and Operating Expenditures (OPEX) were calculated according to the procedure reported in Table S2. Capital investment for infrastructures (electrical, building land improvement and service facilities) are not considered, according to the BIOPOX scenario. Costs of chemicals were taken either from bulk quotation gently provided by Sigma-Aldrich or from WVR and reported in Table S3. A batch schedule has been implemented for operations associated to each unit, accordingly to the procedures reported in previous sections. Major equipment is depreciated assuming 15 years lifetime of the process with an 8% interest rate. When units are not used or more than 20% of overall the batch time, they have been supposed to be shared within the host institution. According to that, the depreciation quote of each equipment was partially charged on the CAPEX of BIOPOX in dependence of the usage time. A 330 days production per year was adopted to take into account maintenance.

Process scale was initially fixed to a working fermenter volume of 2 L and then future scenarios were modelled scaling it up to 200 L. The cost of fermenter was scaled according power law exponents suggested by Kalk and Langlykke (1986). Cost of other equipments were scaled adopting the 6/10 exponent rule (Heinzle *et al,* 2006). Wastewater treatment was fixed to 0.4 € m^{-3} (Heinzle *et al,* 2006).
Further details can be found in supplementary materials.

2.3 Results and discussions

2.3.1 Set-up of culture conditions in shaken flasks

The fungal laccase POXA1b was heterologously produced in the yeast *P. pastoris* investigating the effect of constitutive and inducible expression systems both on the yield and the cost of its production.

In the AOX system methanol works as carbon source as well as inducer of the *AOX1* promoter. In the case of the GAP system both glucose and glycerol were used to support the constitutive expression of POXA1b laccase. Glycerol was tested as alternative carbon source to approach the exploitation of waste glycerol, the main by-product of bio-diesel production (Li *et al.* 2013), in the fermentation process.

The results obtained indicated that, as expected, in the GAP system laccase production is linked to the microbial growth, while in the AOX system a significant laccase production level was achieved upon the inducer addition. Figure 2 displays a comparison of the laccase productivity (volumetric activity per cell biomass) among all the tested conditions. Despite AOX and GAP systems reached a comparable laccase production level, the AOX system exhibited the highest productivity as a consequence of the low cell density achieved.

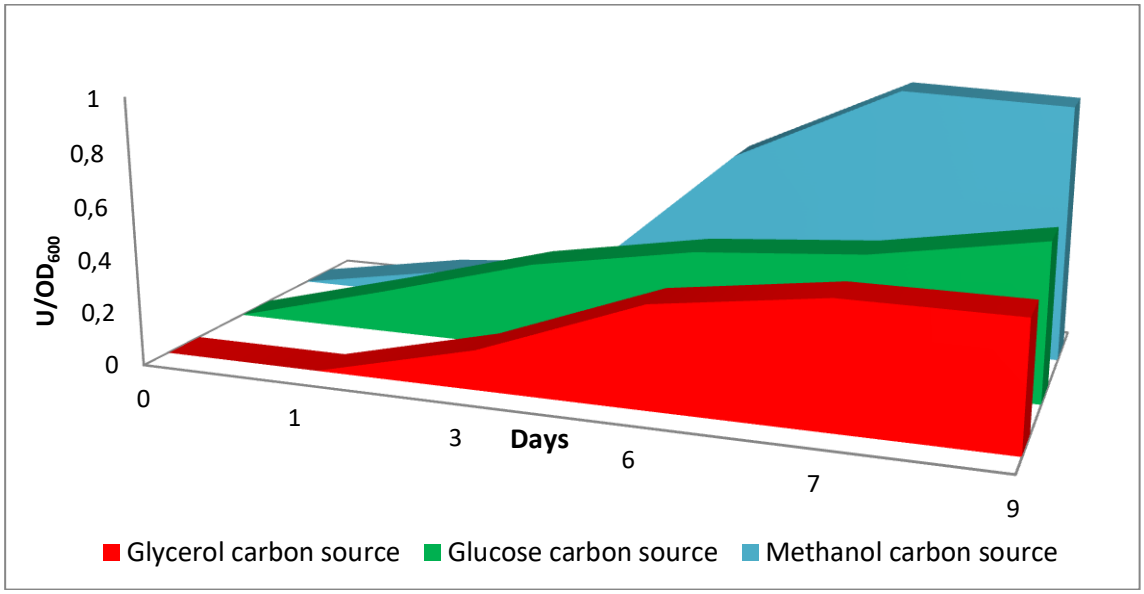


Figure 2. Comparison of different expression system and carbon sources in 1 liter shaken-flask cultivation in BMY medium at 28°C. Standard deviations among three replicates were less than 5%.

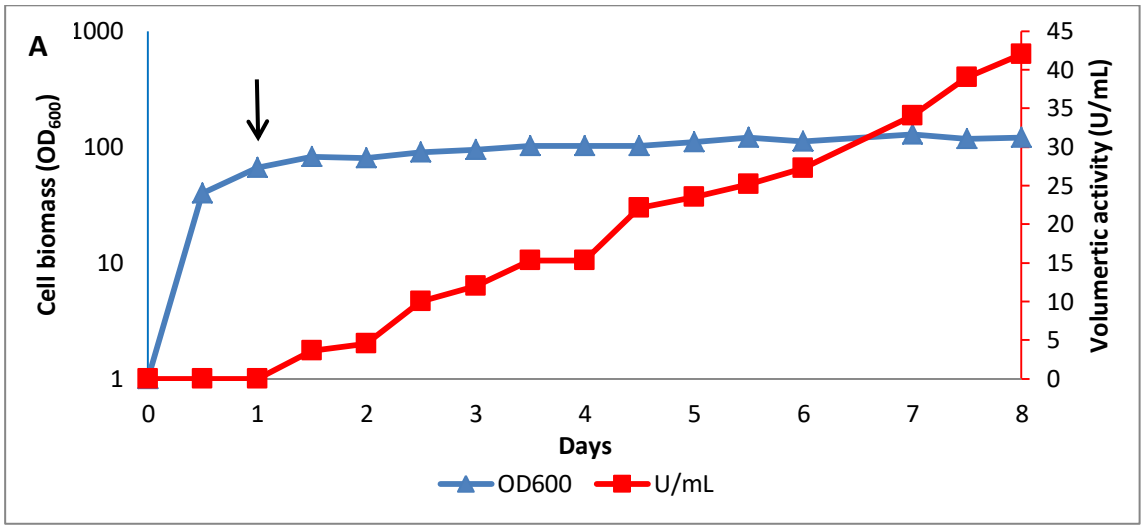
2.3.2 Scale up of recombinant laccase production

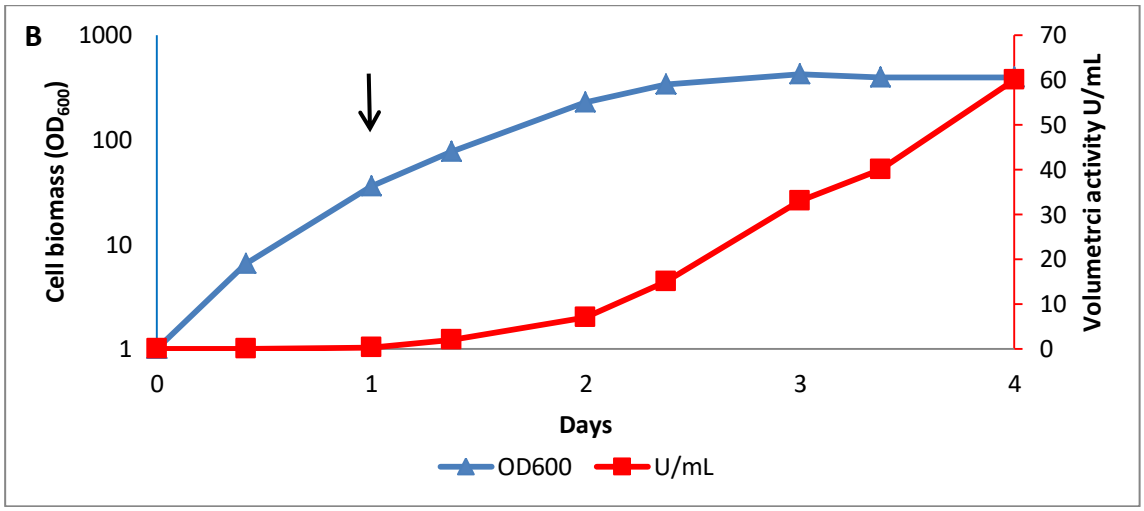
All systems were scaled up in bioreactor in order to confirm the observed productivity trend. Bioreactors were operated in fed-batch mode to promote the highest laccase productivity.

As for the AOX system, within 24 hours from inoculation all the glycerol was consumed by the cells and a cell density of 66 OD_{600} was obtained (Fig. 3A). The end of the batch phase was indicated by a sudden increase in the dissolved oxygen (DO) concentration. After depletion of glycerol, the fed-batch phase started and the induction of rPOXA1b expression was assured by the continuous addition of 1.5% methanol. Adaptation to methanol was noticed by the decrease in the dissolved oxygen (DO) concentration. The highest laccase activity, 42 U/ml (Fig. 3A), was reached after seven days of induction at a final cell density of 120 OD_{600}. This

production level is in agreement with data already reported for laccase production driven by AOX promoter (Kittl *et al.* 2012a; Li *et al.* 2007). Optimization of the fermentation process could further increase laccase yield, as obtained by Hong and coworkers (2002).

When the GAP system was grown in glycerol a cell density of 36 OD_{600} was obtained at the end of the batch phase, after 24 hours (Fig. 3B). Glycerol feeding was assured for three days leading to a laccase activity of 60 U/ml (Fig. 3B) at a final cell density of 390 OD_{600}. The same fermentation process was performed using glucose as carbon source (Fig. 3C). In this case the maximum POXA1b activity and cell density reached after four days of fermentation were ~65 U/ml and 400 OD_{600}, respectively. The GAP promoter has been rarely used for laccase production (Liu *et al.* 2003; Bohlin *et al.* 2006; Kittl *et al.* 2012b; Rivera-Hoyos *et al.* 2015), and, to our knowledge, this is the highest laccase yield obtained from GAP constitutive system.





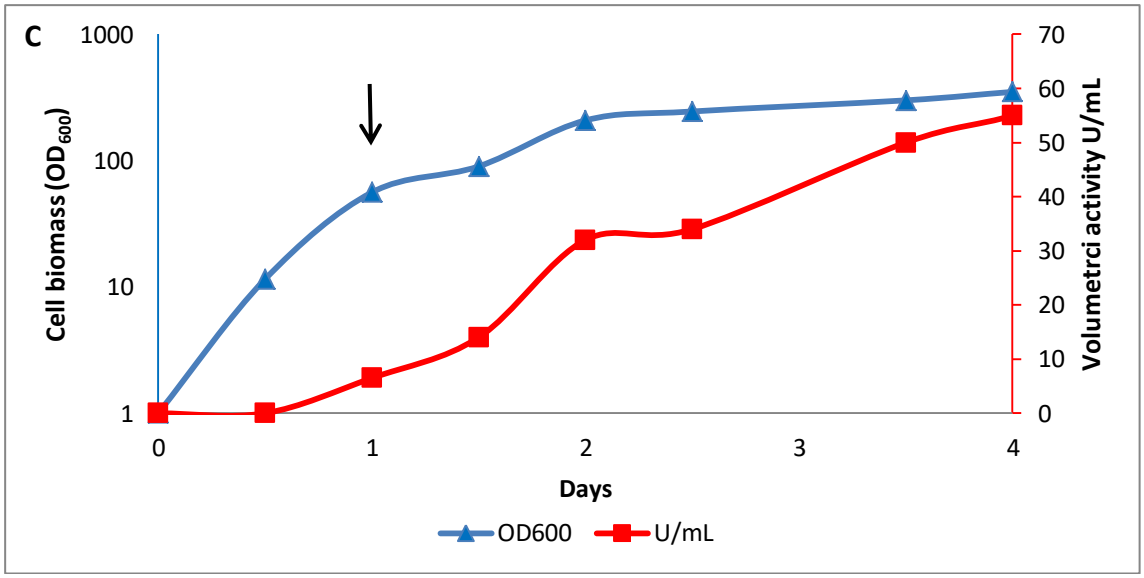


Figure 3. Fed-batch fermentation of expression systems. Standard deviations among three replicates were less than 5%. Arrows indicate the start of the fed-batch phase. **A.** AOX system in MetOH. **B.** GAP system in Glycerol. **C.** GAP system in Glucose.

Data reported in table 1 indicated that AOX system showed the highest laccase productivity respect to GAP, due to the higher biomass production for constitutive system, which decreases the enzyme productivity per liter fermentation medium. The advantage of the GAP systems is that the target protein is produced in shorter time. Furthermore, when this system is grown in the presence of glycerol, a two fold increase in the specific activity (U/mg) was obtained respect to the AOX system, as already reported by Kittl and coworkers (2012). However, when culture broth was concentrated through an ultra-filtration step, the AOX system revealed to be the best performing in terms of both laccase productivity and specific activity. This result indicated the presence of different levels of host cell contaminating proteins, as a function of the used carbon source (Mattanovich *et al.* 2009).
Catalytic parameter towards two substrates of recombinant POXA1b recovered from both systems were comparable to those of other recombinant POXA1b produced in different heterologous hosts (Piscitelli *et al.* 2005; Macellaro *et al.* 2014).
Taking together all these results, it is possible to note that two possible scenarios for POXA1b heterologous production are attainable: AOX system, thanks to its performances (Table 1), and GAP system grown in glycerol, for the shorter fermentation time and for the potential advantage coming from the use of waste glycerol.

Table 1. Comparison between different carbon sources. Standard deviations among three replicates were less than 5%.

Carbon source	Fermentation time (Days)	Laccase productivity (U/OD_{600})	Specific activity broth (U/mg)	Specific activity ultrafiltered (U/mg)
Methanol	8	0.35	230	980
Glucose	4	0.15	230	600
Glycerol	4	0.15	540	660

2.3.3 Techno- economic analysis of POXA1b production

Figure 4 shows the cost breakdown as a function of the fermenter volume for the two selected scenarios. At all the scales the glycerol-based fermentation is more economic than the methanol-based one. The reason is twofold: *i)* glycerol-based batch process takes less fermentation time, obtaining 80 batches per year instead of 46; *ii)* higher POXA1b activity is achieved per batch. At small lab scale the unit production cost of POXA1b is 3.0 € kU^{-1} and 1.61 € kU^{-1} for methanol- and glycerol-based processes, respectively. However CAPEX takes into account more than 40% of the process cost, which is expected at lab scale. When increasing the production with increasing 10 times the operating volume of the fermenter, the cost significantly decreases down to 0.57 € kU^{-1} and 0.32 € kU^{-1} for methanol- and glycerol-based processes, respectively. A further 10 times increase of the fermenter volume (200 L), moving toward a more pilot scale, makes the cost determined mainly by the OPEX and in particular by the cost of chemicals: 0.25 € kU^{-1} and 0.16 € kU^{-1} for methanol- and glycerol-based process, respectively.

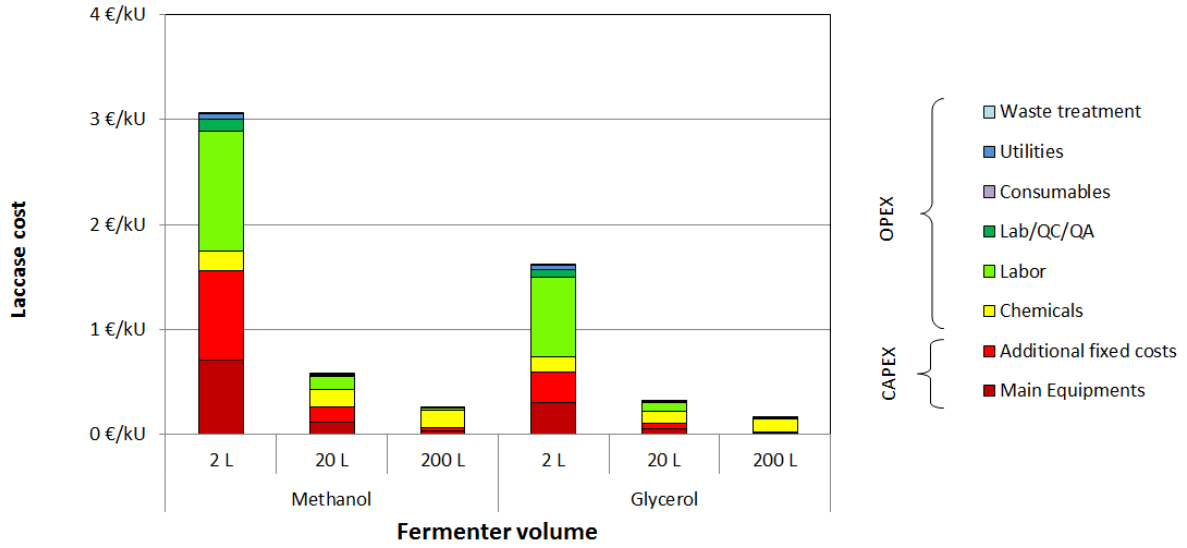


Figure 4. Cost breakdown for methanol and glycerol based process at different scales

This last result gives a clear indication that both processes are competitive at a representative production scale of a small company. As a fact, compared to the prices of the main suppliers of fungal laccase on the market (Rodriguez-Couto 2015), laccases produced by AOX and GAP systems are significantly cheaper.

Moreover, the glycerol-based process has the highest potential for a profitable process due to the high titre of produced enzyme in a shorter time, notwithstanding the lowest specific activity of the enzyme with respect to the methanol-based one.

2.4 Conclusions

Despite the heterologous laccase production offers viable opportunities to tailor enzyme properties, few examples of recombinant laccases are available on the market (Piscitelli *et al.* 2013). This work showed the economic feasibility of POXA1b production.

Two scenarios were proposed based on methanol inducible and constitutive systems, respectively.

Two scenarios were proposed based on methanol inducible and constitutive systems, respectively. The prices of commercially available laccases range from 5 to 200 € kU^{-1} (Rodriguez-Couto 2015). The cost forecast for POXA1b production is around 0.10 € kU^{-1} and is competitive with the current laccase price. In addition, there is still room to lower the process cost using waste glycerol as carbon source.

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2.6 Supplementary materials

Table S 1. Unit cost of electricity, labor and utilities referred to the Italian scenario of industrial production

	Unit cost
Operator labor	28.8 € hr^{-1} *
Electricity	0.16 € kWh^{-1} **
Freon	0.18 € MT^{-1} ***
Chilled water	0.49 € MT^{-1} ***
Hot water	0.062 € MT^{-1} ***
Steam	14.8 € MT^{-1} ***

*source: Bureau of Labor Statistic - www.bls.gov. Lumped cost considering employer contribution, supervision and
**source: Eurostat - http://ec.europa.eu/eurostat/statistics-explained/index.php/Energy_price_statistics
***source: Data are taken from SuperPro databank and linearly scaled by ratio of electricity cost in US and in Italy

Table S 2 – Procedure for estimating CAPEX and OPEX

			Cultivation
TOTAL CAPITAL INVESTMENT	Direct Cost (DC)	Major equipment cost	MEC
		Installation costs	30% MEC
		Instrumentation and control	40% MEC
		Piping	20% MEC
		Insulation	3% MEC
		Electrical	0% MEC
		Buildings	0% MEC
		Land improvements	0% MEC
		Service facilities	0% MEC
	Indirect Cost (IC)	Construction expenses	10% DC
		Engineering and supervision	10% DC
	Other Cost (OC)	Contractor's fee	5% (DC+IC)
		Contingency (Major equipment)	5% (DC+IC)
	Direct fixed capital cost (DFC)		DC+IC+OC
	Working capital		OPEX first month of operation
	Start-up and validation		5% DFC
	Total capital investment		DFC + working capital + Start-up and validation
CAPEX	Depreciation		(DC+IC+OC)/15 years
	Interest		8% of depreciation
	Property tax		2% of depreciation+interest
	Insurance		1% of depreciation+interest
	Purchase tax		5% of depreciation+interest
OPEX	Energy		Calculated from MEC consumption
	Labour		Salaries + Employer's contribution +Supervision
	Raw materials		Calculated from mass balances
	Utilities		Calculated from MEC consumption and mass balances
	Laboratory/Quality control/Quality assurance		10% Labour cost
	Wastewater treatment		Calculated from mass balances
	Consumables		Calculated from MEC design
	Others	Maintenance	5% DFC
		Operating supplies	0.4% (Electricity + Raw materials + Utilities)
		Contingencies	15% (Raw materials + Utilities)
		Overheads	55% (Labour + Maintenance)

Table S 3 – Unit cost of chemicals

Chemical	Unit cost
Yeast nitrogen base **	816 (€/Kg)
Yeast Extract **	169 (€/Kg)
Peptone **	174 (€/Kg)
Biotin *	101,000 (€/Kg)
$CuSO_4\_5H_2O$ **	25 (€/Kg)
NaI **	316 (€/Kg)
$MnSO_4\_H_2O$ **	176 (€/Kg)
$Na_2MoO4\_2H_2O$ **	673 (€/Kg)
H_3BO_3 **	71 (€/Kg)
$CoCl_2$ **	753 (€/Kg)
$ZnCl_2$ **	116 (€/Kg)
$FeSO_4\_7H_2O$ **	71 (€/Kg)
H_2SO_4 (95-98%) **	16 (€/Kg)
H_3PO_4 (85%) **	20 (€/L)
$CaSO_4\_2H_2O$ **	90 (€/Kg)
K_2SO_4 **	55 (€/Kg)
$MgSO_4\_7H_2O$ **	62 (€/Kg)
KOH **	134 (€/Kg)
Glycerol **	11 (€/L)
Antifoam 204*	361 (€/Kg)
Methanol **	3 (€/L)
NH_3 **	11 (€/Kg)
KH_2PO_4 **	140 (€/Kg)
K_2HPO_4 **	170 (€/Kg)
NaOH **	34 (€/Kg)

*Sigma-aldrich
**VWR

CHAPTER 3

Repurposing designed mutants: a valuable strategy for computer-aided laccases engineering. The case of POXA1b

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Repurposing designed mutants: a valuable strategy for computer-aided laccase engineering – the case of POXA1b†

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The broad specificity of laccases, a direct consequence of their shallow binding site, makes this class of enzymes a suitable template to build specificity toward putative substrates. In this work, a computational methodology that accumulates beneficial interactions between the enzyme and the substrate in productive conformations is applied to oxidize 2,4-diamino-benzenesulfonic acid with POXA1b laccase. Although the experimental validation of two designed variants yielded negative results, most likely due to the hard oxidizability of the target substrate, molecular simulations suggest that a novel polar binding scaffold was designed to anchor negatively charged groups. Consequently, the oxidation of three such molecules, selected as representative of different classes of substances with different industrial applications, significantly improved. According to molecular simulations, the reason behind such an improvement lies in the more productive enzyme–substrate binding achieved thanks to the designed polar scaffold. In the future, mutant repurposing toward other substrates could be first carried out computationally, as done here, testing molecules that share some similarity with the initial target. In this way, repurposing would not be a mere safety net (as it is in the laboratory and as it was here) but rather a powerful approach to transform laccases into more efficient multitasking enzymes.

Introduction

Fungal laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are among the best known and greenest biocatalysts in biotechnology.[1] The active core of this class of enzymes consists of four copper ions arranged in two clusters: the T1 copper, placed in the proximity of the protein surface, and the T2/T3 trinuclear cluster (TNC), buried in the protein interior. Substrate oxidation takes place at the T1 site, where H456 and D205 (POXA1b numbering) are the first electron and proton acceptors (if proton transfer is needed).[2] Then, the electron is transferred to the TNC, where oxygen reduction occurs.[3] Laccases have a high potential impact in several biotechnological areas, including pulp and paper, food, furniture manufacturing, and biofuels or nano-biodevices (biosensors and biofuel cells). Furthermore, the use of fungal laccases and/or laccase mediator systems (LMS) for organic synthesis has been studied in depth, ranging from the production of pharmacological compounds (*e.g.* antibiotics, antitumor and antiviral agents) to that of complex polymers.[1,4–6] Such a broad range of applications has led to numerous engineering efforts,[7] including computer-aided strategies,[8,9] aiming at enhancing their activity and/or stability for industrial application. As for specificity, laccases are very promiscuous enzymes, most likely as a consequence of a superficial, shallow binding site which cannot guarantee the optimal positioning for ET for all the putative substrates. Consequently, the T1 pocket could be modified *tout court* to lock the desired substrate in a highly reactive conformation, anchoring it to polar groups. Computational techniques are very promising

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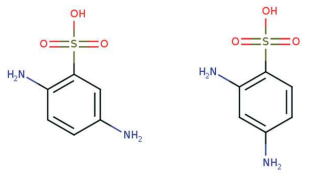


2,5-dabsa 2,4-dabsa

Scheme 1 Chemical structures of 2,5- and 2,4-dabsa.

as they provide an atomistic mapping of both substrate binding and oxidation.[8–10]

In this work, a computational evolution protocol is applied to render the oxidation of 2,4-diamino-benzenesulfonic acid (2,4-dabsa) by POXA1b, a fungal (*Pleurotus ostreatus*) laccase with an unusually high stability at alkaline pH,[11] possible. The substrate of interest, 2,4-dabsa, is a dye precursor just like its constitutional isomer, 2,5-dabsa (Scheme 1). Although 2,4-dabsa is significantly cheaper than 2,5-dabsa, the latter is readily oxidized by POXA1b at alkaline pH and 60 °C while the former is not. Therefore, there is interest in redesigning POXA1b to oxidize 2,4-dabsa, aiming to lower the cost of dye production. Although the engineering effort did not produce an improved variant toward the oxidation of 2,4-dabsa, the mutant variants display a significantly increased ability to oxidize three typical substrates of laccases. Molecular calculations suggest that such an improvement is the consequence of specific enzyme–substrate interactions accumulated during computational evolution. The consequences for computer-aided laccase engineering and mutant repurposing are discussed.

Experimental section

Homology modeling and system setup

The POXA1b structure was generated through Prime homology modeling,[12,13] automatically selecting 1GYC.pdb (60% sequence identity) as a template. POXA1b is characterized by a longer C-terminal tail, which could be placed outside (**1**) or inside (**2**) the oxygen cavity (Fig. 1). The structure of **2** was taken from a previous work.[14] The POXA1b structures (**1** and **2**) were prepared with Protein Preparation Wizard.[15] The protonation states of titratable residues were generated with PROPKA[16] and double-checked with the H++ server,[17] simulating pH 8.

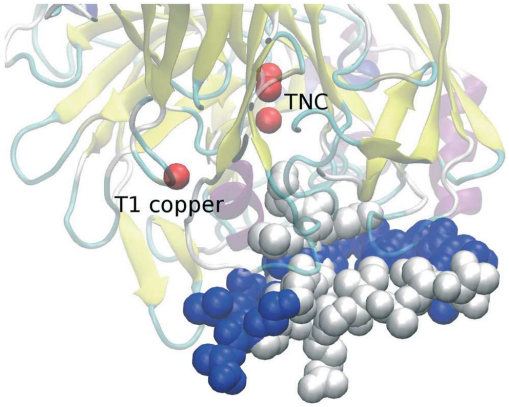


Fig. 1 Conformations of **1** (blue VDW spheres) and **2** (white VDW spheres) obtained from homology modeling.

Molecular dynamics

Molecular dynamics (MD) simulations were performed with GROMACS[18] for **1** and **2** to refine the initial homologous structures. The enzymes were solvated with a 10 Å layer of water molecules in a dodecahedral box, adding enough ions for neutralization and a 0.15 M NaCl buffer. The systems prepared were equilibrated as follows: i) solvent minimization; ii) system minimization; iii) 200 ps system warm up from 15 to 298 K at constant volume; iv) 200 ps at constant volume and 298 K; and v) 200 ps at 298 K and 1 bar. Then, a 200 ns production run was performed at 298 K and 1 bar for each model. The AMBER99 force-field[19] and the SPC explicit water model[20] were used. The copper ions, modeled in their reduced (+1) state (to attenuate charge repulsion in the TNC), the coordinating atoms and their nearest neighbours were restrained to their initial positions with stiff 10 000 kJ mol^{-1} nm^{-2} harmonic constraints. The temperature was regulated with velocity rescaling[21] with a relaxation time of 0.1 ps, and the pressure was controlled with a Parrinello-Rahman barostat[22] with isotropic coupling and a relaxation time of 2.0 ps. The LINCS algorithm[23] was employed to constrain all bond lengths, allowing a time step of 2.0 fs. A 10 Å cutoff was used for non-bonded interactions together with the particle mesh Ewald method.[24] It is well known that homology models can fall apart when subjected to MD,[25] thus a 1000 kJ mol^{-1} nm^{-2} harmonic constraint was applied to the secondary structure elements (helices and sheets) for the first 40 ns and lowered to 10 kJ mol^{-1} nm^{-2} for the rest of the simulation.

PELE sampling

The laccase–substrate conformational space, for the wild type (wt) and mutant variants and for all the substrates mentioned in the text, was sampled with PELE,[26,27] our in-house software. The substrate, initially placed in front of the T1 cavity, was randomly translated and rotated within 15 Å of the T1 copper. The laccase was perturbed according to an anisotropic network model (ANM) applied to all the alpha carbons in the system. Residues within 10 Å of the substrate were minimized after both the substrate and the enzyme were perturbed. Throughout the study, (PELE) binding energies refer to protein–substrate interaction potential energies.

PELE design

The initial conformation for the design was selected from the wt PELE sampling among the structures that exhibit catalytic contacts with H456 and D205, the first electron and proton acceptor, respectively. Then, every residue within 10 Å of the substrate was mutated to all of the possible remaining 19 amino acids; mutated residues included: T160, G161, V162, P163, H164, C204, S206, N207, A239, N263, S264, F331, P333, A336, A391, G392, P393, P395, W455, P510, and L511. The ligand was perturbed with very small translations and rotations while the protein backbone was subjected to small ANM displacements. Every residue within 10 Å of the

substrate was energy minimized. The simulation of each mutant (and the wild type) lasted 1 hour, accumulating ~30 Monte Carlo steps (~20 accepted). The enzyme–substrate interaction energy was used to score each mutant, while keeping an eye on the substrate's solvent accessible surface area (SASA). An example of input file for both PELE sampling and design is available in the ESI† and can be uploaded to the PELE server[28] to reproduce the results in this paper.

Treatment of substrate and metal centers in PELE

The substrates' geometries were optimized at the M06/6-31G* level of theory, modeling the solvent with the Poisson–Boltzmann model, using Jaguar.[29] The atomic ESP charges were calculated from the optimized structure and used for PELE. The OPLS parameters were assigned to the ligand with Schrödinger's hetgrp_ffgen utility and a rotamer library was generated with the in-house PELE's scripts using Macromodel.[30] The copper ions and their coordination atoms plus their nearest neighbors were constrained during PELE sampling with 200 kcal mol^{-1} $Å^{-2}$ harmonic constraints on their atomic positions.

Materials

ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), 2,4-dabsa, sinapic acid, syringic acid, and caffeic acid were purchased from Sigma-Aldrich. All chemicals were of reagent-grade purity. Expression vectors and yeast strains were purchased from Biogrammatics, Ltd (Las Palmas Dr, Carlsbad, CA, USA).

Construction and screening of POXA1b expressing mutants

The QuikChange site-directed mutagenesis kit was purchased from Agilent Technologies. Synthetic oligonucleotides were produced by Eurofins Genomics. Plasmid purification kits were purchased from Qiagen, Inc. A synthetic gene encoding for *Pleurotus ostreatus* POXA1b was designed and optimized according to *P. pastoris* codon usage. The gene product was restricted with BsaI and ligated to the corresponding site of the pJGG_αkR vector in-frame with the α-factor (signal peptide) under the control of the constitutive GAP promoter, yielding the recombinant plasmid pJGGαKR_POXA1b. Mutagenesis of the wild type POXA1b cDNA within the plasmid pJGGαKR_POXA1b was performed by following the instructions provided in the QuikChange site-directed mutagenesis kit. The sense oligonucleotides used for mutagenesis are as follows:

5′ CCTTGTCTTTGACTGGTGTTCCACACCCAGACTCCAC 3′ for Val162Ser,

5′ CCTTGTCTTTGACTGGTGTTCCACACCCAGACTCCAC 3′ for Val162His,

5′ CTTGAACTTCGCTTTCGACCCTGCTACTGC 3′ for Phe331Tyr, and

5′ CGCTTACGACCCTGCTACTGCTTTGTTCACTGCTAACAACC 3′ for Ala336Asn.

The complementary antisense oligonucleotides also necessary for the mutagenesis are not shown. The mutagenized recombinant plasmids (pJGGαKR_V162S_F331Y_A336N and pJGGαKR_V162H_F331Y_A336N) were then used to transform ultracompetent cells as described by the manufacturer. Recombinant plasmids were purified from 5 mL overnight cultures using the Qiagen miniprep system. The presence of the correct mutation was confirmed in all constructs by DNA sequencing (Eurofins Genomics). The mutagenized plasmids were linearized by *BsiWI* and transformed into *P. pastoris* BG-10 by electroporation.[31] The cell suspension was spread on YPDS (10 g l^{-1} yeast extract; 20 g l^{-1} bacto tryptone; 20 g l^{-1} glucose; 182.2 g l^{-1} sorbitol) plates containing 900 μg ml^{-1} G418 and incubated for 3–7 days at 28 °C until colony formation. The colonies were collected, inoculated in Minimal Dextrose medium (MD) (13 g l^{-1} yeast nitrogen base with ammonium sulfate w/o amino acids, 4×10^{-4} g l^{-1} biotin, 20 g l^{-1} glucose), and grown at 28 °C on a rotary shaker (250 rpm). The colonies were then streaked on solid MD medium containing the laccase chromogenic substrate ABTS (13 g l^{-1} yeast nitrogen base with ammonium sulfate w/o amino acids; 4×10^{-4} g l^{-1} biotin; 20 g l^{-1} glucose, 0.6 mM $CuSO_4$ and 0.2 mM ABTS) to verify the expression of the recombinant proteins as well as on YPDS (10 g l^{-1} yeast extract; 20 g l^{-1} bacto tryptone; 20 g l^{-1} glucose; 182.2 g l^{-1} sorbitol) with 900 μg ml^{-1} G418. Recombinant pJGGαKR_V162S_F331Y_A336N and pJGGαKR_V162H_F331Y_A336N cultures selected from solid screening assay were inoculated in 20 ml BMMY (13 g l^{-1} yeast nitrogen base with ammonium sulfate without amino acids; 10 g l^{-1} yeast extract; 20 g l^{-1} peptone; 100 mM potassium phosphate, pH 6.0; 4×10^{-4} g l^{-1} biotin; 0.5% methanol) medium in a 100 ml shake flask. These cultures were grown for 8 days at 28 °C on a rotary shaker (250 rpm).

Assay of laccase activity

Culture aliquots were collected daily and assayed for cell density and extracellular laccase activity. Laccase activity in the culture supernatant was assayed at room temperature, monitoring the oxidation of ABTS at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}): the assay mixture contained 2 mM ABTS and 0.1 M Na-citrate buffer, pH 3.0. Purified laccase activity towards 2,4-dabsa was assayed at 60 °C temperature, monitoring the oxidation of 2,4-dabsa in the visible spectrum. The mixture contained 25 mM 2,4-dabsa and 0.1 M Tris–HCl buffer, pH 8.0.

Production and purification of POXA1b and mutated laccases

Recombinant *P. pastoris* cells expressing the two POXA1b mutants were inoculated in 50 ml BMMY medium in a 250 ml baffled shake flask. These precultures were grown for one day at 28 °C on a rotary shaker (250 rpm), then a volume of the suspension sufficient to reach a final OD600 value of 1.0 was used to inoculate 1 liter shake flasks containing 250 ml of BMMY medium. Cells were grown on a rotary shaker (250 rpm) at 28 °C and 3.75 ml of methanol was added to the

culture each day of growth to induce protein expression. The cultures were concentrated to 20 ml in a Pall multi-cassette system (10 kDa cutoff membrane). The samples were applied to an anion-exchange column (Hi-Prep 16/10) equilibrated with 20 mM Tris–HCl (pH 8.0) at a flow rate of 0.7 ml min^{-1}. The retained proteins were eluted with a gradient of 20 mM Tris–HCl (pH 8.0) plus 1 M NaCl. The chromatographic fractions peaks containing laccase activity were pooled, concentrated and dialysed against 20 mM Tris–HCl (pH 8.0). The active fractions were brought to 100% ammonium sulfate saturation and then centrifuged at 9300*g* for 10 min. The precipitated proteins were dissolved in 20 mM Tris–HCl (pH 8.0) and dialysed against the same buffer. A volume of 5 ml of dialyzed protein solution was transferred to a HiLoad 16/600 Superdex 200 pg column, equilibrated with 20 mM Tris–HCl (pH 8.0) plus 0.2 M NaCl, and eluted using the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions were collected and dialysed against 20 mM Tris–HCl (pH 8.0). The dialysed samples were assayed for laccase activity and protein concentration. The specific activities of both purified enzyme variants were comparable to that of the wild-type.

Biochemical characterization of laccase variants

The oxidation of substrates by the purified laccase was determined spectrophotometrically (UVIKON 930, Kontron Instruments) at the specific wavelength of each substrate. The assays were performed by measuring the increase in the Abs 470 nm for syringic acid (ε = 0.5 mM^{-1} cm^{-1}), Abs 470 nm for caffeic acid (ε = 1.8 mM^{-1} cm^{-1}) and Abs 595 nm for sinapic acid (ε = 0.3 mM^{-1} cm^{-1}). The molar extinction coefficients of the three fully oxidized substrates were determined from their corresponding calibration curves. The kinetic constants K_M and V_{max} of the enzymes were determined using a Michaelis–Menten plot with syringic and caffeic acids. The kinetic constants K_i, K_M and V_{max} of the enzymes were determined in the case of sinapic acid using the equation reported below.[32] All substrates were dissolved in 20 mM Tris–HCl (pH 8.0).

$$V = \frac{V_{max}[S]}{K_M + [S] + [S]^2/K_I} \quad (1)$$

Results and discussion

Refinement of POXA1b models

The presence of a protruding C-terminal tail, compared to other laccases from basidiomycetes, has been previously observed in POXA1b.[33] According to homology modeling, such a tail could be placed either outside (**1**) or inside (**2**) the oxygen cavity (Fig. 1). MD simulations were performed to refine the models and establish whether **1** or **2** (or none of them) is the best representation of the system. Simulation of **1** converged in ~50 ns (Fig. 2, simulation stopped at ~160 ns), keeping the C-terminal away from the oxygen cavity (Fig. 3). In contrast, **2** is rather unstable (Fig. 2), resulting in the escape of the C-terminal from the protein interior, roughly matching **1** (Fig. 2). It follows that, according to the simulation, the C-terminal is more stable on the protein surface than in the interior. Moreover, the C-terminal of **1** directly interacts with the active site, adding a further degree of complexity to the design. Such a finding makes POXA1b's

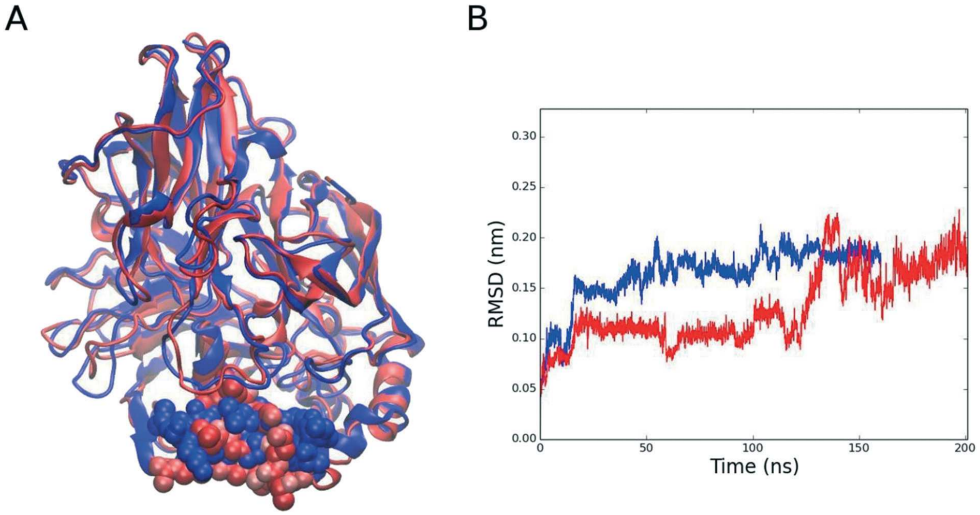


Fig. 2 A) Final snapshot from the MD simulation of **1** (blue) and **2** (red). The C-terminal tail is represented by VDW spheres. B) Heavy atom RMSD plots from the MD simulations of **1** (blue) and **2** (red).

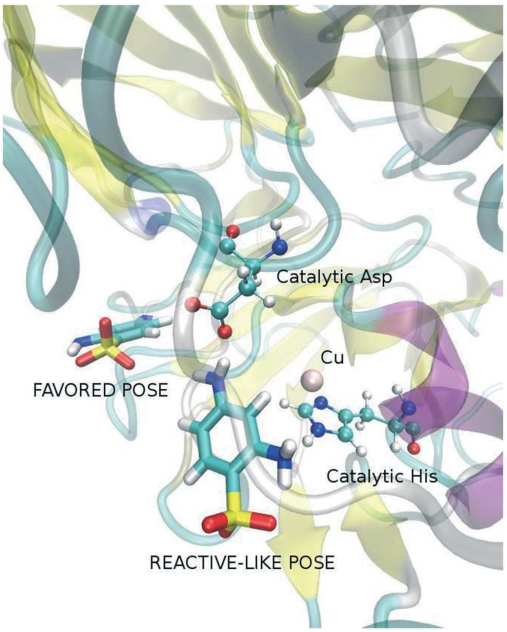
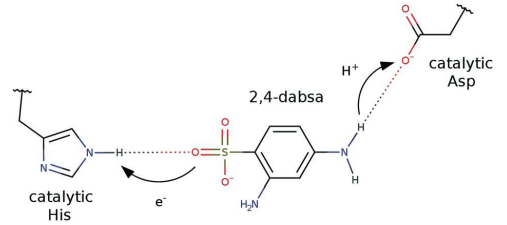


Fig. 3 Favored (best binding) and reactive-like (catalytic contacts) poses from PELE sampling for 2,4-dabsa.

C-terminal somewhat different with respect to that observed for *Melanocarpus albomyces* laccase, which is also resistant at alkaline pH. Its C-terminus is located in the proximity of the oxygen channel, obstructing small molecules' passage.[34] Nonetheless, the characterization of the truncated POXA1b mutants highlighted the key role of the C-terminus tail in protein stability at alkaline pH.[33]

Computational comparison of 2,4- *vs.* 2,5-dabsa

Binding of 2,4- and 2,5-dabsa in the T1 pocket was simulated with 96 independent 48 hour PELE runs. Inspection of the structures within 5 kcal mol^{-1} of the lowest PELE binding energy value (within 12 Å) reveals that no catalytic contact (*i.e.* H-bonds with H456 and D205, Scheme 2) is engaged with 2,4-dabsa (Fig. 3). In order to find a pose that displays catalytic interactions, it is necessary to climb the PELE binding energy ladder by ~30 kcal mol^{-1} (Fig. S3;† note that this value is a protein–ligand interaction energy, not a proper binding energy). Such an energy difference poses a great obstacle to oxidation, since the enzyme severely selects unproductive poses, *i.e.* with non-optimal electron tunnelling between the substrate and the T1 copper, slowing 2,4-dabsa oxidation down. The same analysis revealed that 2,5-dabsa displays such catalytic contacts with H456 in 13% of the low-lying binding energy conformations (Fig. S4†), but never with D205. Therefore, based on enzyme–substrate sampling, 2,4-dabsa binds unproductively to the T1 site compared to 2,5-dabsa; such a distinct binding preference might be ascribed to the different orientations of the amino groups in the benzene ring, which determines diverse H-bonding patterns with the enzyme.



Scheme 2 Expected catalytic contacts.

A further cause for the different reactivities of the two isomers might lie in their different propensities for oxidation. This possibility was addressed by comparing the energy of the HOMO orbitals of 2,4- and 2,5-dabsa. According to Koopmans' theorem, the negative of the HOMO energy of a molecule corresponds to its ionization energy,[35] assuming no subsequent orbital relaxation (and neglecting electronic correlation). The geometry optimization of both isomers at the RHF/6-31G* level predicts the ionization energy of 2,4-dabsa to be larger by ~175 meV. Thus, not only does 2,4-dabsa have a worse interaction with the enzyme but it is also harder to oxidize than 2,5-dabsa.

Based on the above enzyme–substrate analysis, and following recent studies indicating the importance of the substrate binding event in laccases,[8–10] the engineering efforts were focused on the formation of (in principle) more reactive conformations of the Michaelis complex. Such a classical study does not require quantum chemical calculations, while tuning 2,4-dabsa oxidability inside the T1 pocket would.[8] Moreover, the outcome of this engineering effort would be a better starting point to target the oxidability of 2,4-dabsa. Finally, increasing the redox potential of the T1 copper is not straightforward and it does not guarantee success.[36–39] Therefore, the reactive-like pose depicted in Fig. 3 was selected as the initial template for the design, aiming at improving the enzyme–substrate interaction energy of such a binding mode, hence its statistical importance in the active site.

Design of a more reactive Michaelis complex

An evolutionary strategy was employed to accumulate beneficial mutations at each computational mutagenesis round (Fig. 4). During an evolution round, a mutation was considered beneficial if the binding energy improved by 5 kcal mol^{-1} or more; then, one candidate was selected and used as the starting template for the next round. It should be noted that, at each of these rounds, the mutants showing improved PELE binding energy were all visually inspected, bringing

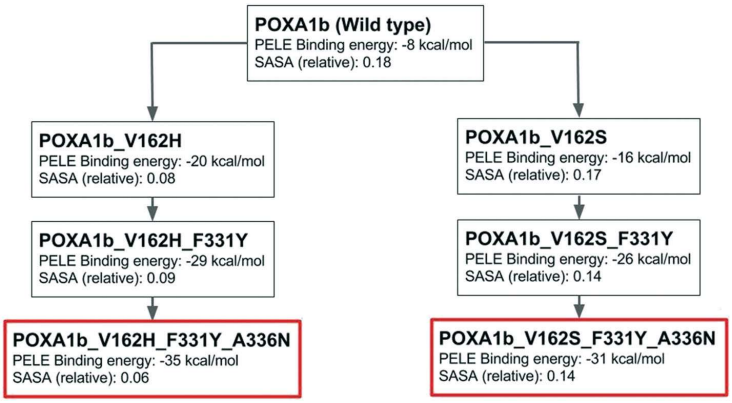


Fig. 4 Evolutionary strategy and results of POXA1b design toward 2,4-dabsa oxidation.

chemical intuition into play. In the first round, two mutations looked promising due to a great increase in PELE's binding energy and, in one case, a significant drop in SASA (hence a possible decrease in reorganization energy[40]): V162H and V162S (Fig. 4).

Although other mutations present interesting values (Table S1†), they were discarded in favor of V162H and V162S for one of the following reasons: i) they involved Gly/Pro (as the starting amino acid), which might trigger important loop conformational changes due to their flexibility/rigidity which cannot be captured at this level of sampling (they would need extensive sampling to confirm their reliability); ii) unless they introduce outstanding improvement, positive charges are avoided as they might inhibit oxidation; iii) they were located on the C-terminal tail, whose conformation is the most fragile aspect of the model; and iv) visual inspection suggested caution.

After the first round, computational evolution was branched, further designing both the V162H and V162S variants (Fig. 4). In the second round of both V162H and V162S branches, F331Y was chosen, while in round 3, A335N was selected for both branches as well. Computational evolution was stopped at this point.

In both branches, three new H-bonds with the sulfonate group of 2,4-dabsa were introduced (Fig. 5), increasing the binding energy by more than 20 kcal mol^{-1} (SASA is also significantly reduced in the V162H branch). Therefore, the evolved active sites should be able to select the substrate in a reactive conformation with much higher frequency. In order to test this hypothesis, a new PELE extensive sampling run was carried out for the evolved triple mutants, as previously done for the POXA1b wt; the catalytic and designed contacts were then checked in the structures within 5 kcal mol^{-1} of the lowest binding energy value. Nicely, for the V162S branch, all the designed H-bonds and the H456–sulfonate contact were retained in all the binding modes, while the D205–amino contact was formed in 40% of the cases. In contrast, this last contact is never formed in the V162H branch. On top of that, although H456 and N336 interact with the sulfonic group, the other designed contacts did not form as planned: the side chains of Y331 and H162 interact with the amino groups instead (Fig. S1†). Still, the resulting binding

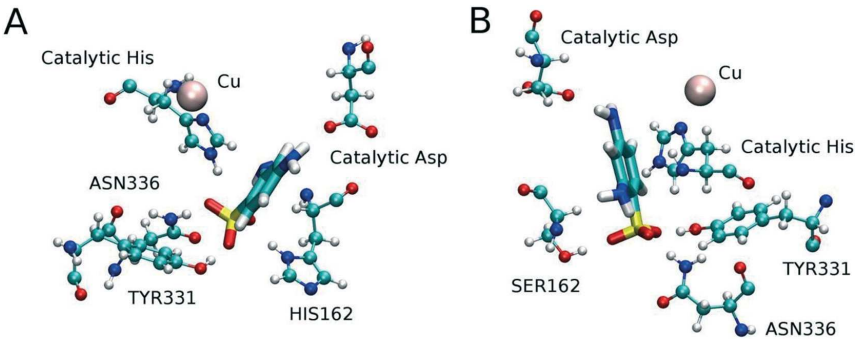


Fig. 5 Structural model of the designed triple mutants. A) V162H branch; B) V162S branch.

mode is more reactive than in POXA1b (Fig. 3): an efficient through bond (sulfonate–H456) electron tunnelling path is set, while the aminic proton could be abstracted by a water molecule (if proton transfer occurs simultaneously), although it is not the optimal path. Therefore, it was decided to test also the V162H branch triple mutant in the lab.

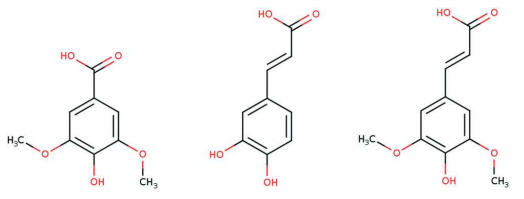
Experimental validation, rationalization of results and mutant repurposing

Regardless of the promising mutant validation with PELE sampling, the evolved variants were inactive towards 2,4-dabsa, just as POXA1b. The possible reasons behind this unsatisfying result are as follows: i) the homology model is inaccurate in the active site description due to the presence of the C-terminal and ii) 2,4-dabsa is not significantly oxidized by the T1 copper (*i.e.* the ET driving force is positive and too large). While the first point is difficult to verify, the ~175 mV difference in ionization energy between the dabsa isomers suggests that it might be a substrate oxidability issue. Further analysis of these possibilities was studied by testing additional ligands.

Although the designed variants failed to oxidize the target substrate, their putative ability to lock in place 2,4-dabsa's sulfonic group could be exploited to improve the oxidation of other negatively charged substrates. As reported in the literature,[10] the introduction of polar amino acids in the T1 pocket enhances its binding interaction with sinapic acid, a negatively charged substrate (Scheme 3). Since the presented computational evolution effort yielded a more polar T1 site, the oxidation of sinapic acid was characterized with experimental methods, aiming at verifying if the mutant variants can oxidize it better. In addition, caffeic and syringic acids (Scheme 3) were also selected for laboratory characterization in terms of their chemical similarity to sinapic acid. These two molecules are representative of two different classes of substances, hydroxycinnamates and trihydroxybenzoic acid, with different industrial applications.[41,42] By testing and simulating these substrates, which are regularly oxidized by laccases, it is possible to assess if the designed binding moiety is effective and leads to activity improvement.

As can be seen in Tables 1–3, the efficiency constant (k_{cat}/K_M) of the triple mutants is higher than that of POXA1b for all the substrates (Tables 1–3). Interestingly, sinapic acid substrate inhibition is also attenuated in the evolved variants, as reflected by the larger inhibition constant (K_i) values, while an overall tendency of increasing K_M and k_{cat} can be observed (Table 3). Since the kinetic data for this substrate are obtained from a substrate inhibition model (the substrate binds unproductively to the enzyme while hindering the formation of productive enzyme–substrate adducts[32]), the trend of the kinetic constants implies that either the unproductive poses are destabilized or the productive conformations are stabilized in the mutant. The second hypothesis could be true despite the larger K_M values, since this constant would increase with k_{cat} in a steady-state kinetic regime.[43]

In order to obtain a molecular mechanism for the increase in substrate oxidation, the binding of these three substrates in the T1 pocket of POXA1b and its evolved variants was simulated with PELE. The objective was to verify whether such improved catalytic performance is related to the larger population of conformations in which the carboxyl group (which is deprotonated at pH 8) is H-bonded to H456. As a matter of fact, POXA1b never shows H-bonds between H456 and the substrates, forcing an inefficient through-space electron tunnelling between the substrate and the T1 center. On the other hand, H-bonds between the substrate and H456 have a significant population for every mutant–substrate pair, which ranges from 29 to 81% (Tables 1–3) among the structures within 5 kcal mol^{-1} of the binding energy minima and within 10 Å of the T1 copper. This contact facilitates a through-bond electron tunnelling, which greatly improves electron transfer. Remarkably, the sinapic acid–V162S branch mutant pair, the one that experiences the largest improvement in k_{cat} (~15 fold, Table 3), exhibits the largest population of this contact (81%) and forms all the designed H-bonds in 61% of the cases (Fig. 6). Finally, it is worth noting that no contact is formed between the substrate and D205 in any variant–substrate pair, meaning that such a contact is not indispensable (water molecules can assist deprotonation), although design toward the instauration of such interaction could further improve oxidation (especially due to the farther vicinity of a negative charge that could shift up the HOMO energy of the substrates).



Scheme 3 Chemical structures of syringic, caffeic and sinapic acids.

Consequences for laccase design

Although the initial design was not successful, the repurposing effort suggests (both in its experimental and computational validation) that the non-specific reaction site of laccases can be a valuable template to build more specific interactions with a target substrate. This can be implemented with the presented computational methodology, increasing the affinity of the substrate for the enzyme in a highly reactive conformation. Nonetheless, laccase design proved to be a delicate multidimensional problem which requires to always take an electron transfer driving force into account. As seen, if a target substrate is poorly oxidizable, the electron transfer driving force might become a crucial design parameter, tunable as shown recently.[9]

Table 1 Experimental kinetic constants and population of structures exhibiting catalytic carboxyl-H456 H-bonds during PELE sampling for the oxidation of caffeic acid by POXA1b and the designed triple mutants

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	Sub–H456 H-bonds (%)
POXA1b	3.52 ± 0.53	1262 ± 60	360 ± 60	0
V162H branch	0.47 ± 0.11	1122 ± 54	2805 ± 54	50
V162S branch	3.65 ± 0.57	4184 ± 35	1162 ± 35	29

Table 2 Experimental kinetic constants and population of structures exhibiting catalytic carboxyl-H456 H-bonds during PELE sampling for the oxidation of syringic acid by POXA1b and the designed triple mutants

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	Sub–H456 H-bonds (%)
POXA1b	1.22 ± 0.11	1775 ± 87	1400 ± 87	0
V162H branch	1.35 ± 0.27	2700 ± 54	2000 ± 54	60
V162S branch	0.82 ± 0.25	6997 ± 87	8000 ± 87	50

Table 3 Experimental kinetic constants and population of structures exhibiting catalytic carboxyl-H456 H-bonds during PELE sampling for sinapic acid by POXA1b and the designed triple mutants

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})	K_i (mM)	Sub–H456 H-bonds (%)
POXA1b	0.13 ± 0.05	6681 ± 100	51 392 ± 100	1.23 ± 0.09	0
V162H branch	0.41 ± 0.03	39 113 ± 500	95 397 ± 500	2.44 ± 0.09	66
V162S branch	0.67 ± 0.02	97 126 ± 600	144 964 ± 600	1.49 ± 0.08	81

Repurposing should not be a mere safety net (as it was in this case) but rather an effective strategy for industrial enzyme production. Computational design toward a given substrate could be accompanied on-the-fly by the *in silico* evaluation of a database of putative substrates, possibly selecting a subset based on target molecular similarity. In this way, several processes could be improved at the cost of one or a new design line for a particularly promising substrate could be opened. Ideally, variant search should not be fossilized on a fixed laccase either. These enzymes present great diversity in their T1 pockets that could be exploited to design the best host as much as possible for a (class of) substrate(s). Even if a very promising laccase did not function under the desired working conditions (pH, temperature, presence of ions and/or co-solutes, *etc.*), the enzyme could be designed to adapt to them. Therefore, a future where several laccases are computationally evolved against many substrates can be envisioned. Pushing further, efficient chimeric laccases might be designed by conveniently stitching portions of proteins from different laccases with computational techniques.[44]

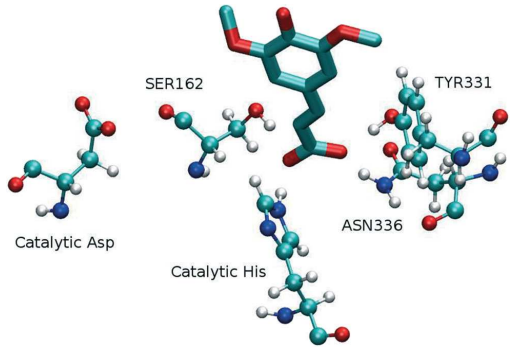


Fig. 6 PELE sampling result for the binding of sinapic acid in the active site of the V162S branch triple mutant.

Conclusions

This work showed that the T1 site is a promiscuous template to increase activity toward specific target substrates, through the design of stabilizing enzyme–substrate interactions in a highly reactive conformation. The experimental and computational validation of mutant repurposing suggests that this task could be achieved with the computational protocol proposed in this work. Nonetheless, it should be borne in mind that laccase design is an intricate multidimensional problem in which the ET driving force is an important factor. Likely due to the superficial and shallow nature of the T1 site, repurposing designed mutants to oxidize molecules that share some degree of similarity with the target proved to be effective. In the future, repurposing might be carried out computationally and subsequently validated experimentally, transforming laccases into more efficient multitasking enzymes.

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CHAPTER 4

Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification

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Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification

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ABSTRACT

The clarification step represents, in fruit juices industries, a bottleneck process because residual phenols cause severe haze formation affecting juice quality and impairing customers acceptance. An enzymatic step can be efficiently integrated in the process, and use of immobilized enzymes entails an economical advantage. In this work, covalent immobilization of recombinant POXA1b laccase from *Pleurotus ostreatus* on epoxy activated poly(methacrylate) beads was optimized thanks to a Response Surface Methodologies approach. Through regression analysis the process was well fitted by a quadratic polynomial equation (R^2 = 0.9367, adjusted R^2 = 0.8226) under which laccase activity reached 2000 ± 100 U g^{-1} of beads, with an immobilization efficiency of 98%. The immobilized biocatalyst was characterized and then tested in fruit juice clarification reaching up to 45% phenol reduction, without affecting health-effective flavanones content. Furthermore, laccase treated juice displays an improved sensory profile, due to the reduction of vinyl guaiacol, a potent off-flavor possessing a peppery/spicy aroma.

1. Introduction

According to the "European fruit juice association" (www.aijn.org), EU fruit juice and nectars consumption is forecast to stand at 10.3 billion liters by 2017. The ongoing consumer health and wellness trend in many countries offers strong potential for increasing this market, due to the well-established beneficial effects associated to fruit juices consumption (Bharate & Bharate, 2014). Polyphenols in fruit juices are a natural source of antioxidants, and are responsible of the reported health benefits (Agcam, Akyıldız, & Akdemir Evrendilek, 2014). However, the same compounds are also the main factors involved in maderization process causing turbidity, color intensification, aroma and flavor alteration and formation of haze or sediments, affecting final product shelf life and consumer perception (Pezzella, Guarino, & Piscitelli, 2015). In order to reduce the impact of this phenomenon on the beverage products and to stabilize fruit juices, industries commonly use clarification processes through physical–chemical adsorbents and/or filtration technology. One disadvantage of these techniques is that processed juices are not always stable, but rather tend to produce pronounced haze and enzymatic and non-enzymatic browning, caused by reactive phenolic compounds that cannot be removed (Friedman, 1996). Research has been concentrated, in recent years, to find effective and economical ways to reduce this phenomenon, with enzymes continuously gaining importance. Among enzymes useful within this process, several authors have proposed the use of laccases as stabilizing agents, due to their ability to oxidize most of the phenols present in juices (Neifar et al., 2011 and Gassara-Chatti et al., 2013) causing their polymerization and subsequent ease of removal.

Immobilization provides an excellent base for enzymes exploitation by increasing their reusability, enhancing their structural and catalytic stability in different environmental conditions, and reducing product inhibition (Sheldon, 2007). Apart from being affordable, immobilization generates continuous economic operations, automation, high investment/capacity ratio and recovery of product with greater purity (D'Souza, 1998). Several methods are used for enzyme immobilization and various factors influence the performance of the immobilized enzymes (Pezzella, Russo, Marzocchella, Salatino, & Sannia, 2014). The choice of the most suited method and the chemical nature of support material clearly depends on the application that enzyme is devoted to. Particularly, covalent binding is the most widely applied method in industrial

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applications due to several advantages including improved operational stability, robustness and reusability. The stabilization provided by covalent bonding is usually counterbalanced by partial enzyme deactivation. This negative effect can be mitigated by carefully optimizing the immobilization conditions in order to maximize the ratio between immobilized enzyme activity and activity of the primary enzyme solution.

In this work, covalent immobilization of the recombinant fungal laccase POXA1b (rPOXA1b) from the edible fungus *Pleurotus ostreatus* (Giardina et al., 1999) on epoxy activated poly(methacrylate) supports (Mateo et al., 2002 and Mateo et al., 2007) was investigated and the solid bio-catalyst was tested in fruit juices treatment. Laccases are widespread enzymes able to oxidize a wide range of phenolic substrates, using only molecular oxygen as cofactor and generating water as unique by-product. Due to its high redox potential and belonging to an enzymatic system of an edible fungus (Macellaro, Pezzella, Cicatiello, Sannia, & Piscitelli, 2014), rPOXA1b laccase has a great potential exploitability in food and beverage industries. The successful application of laccases in these fields require production of high amounts at reduced costs (Osma, Toca-Herrera, & Rodríguez-Couto, 2010). Concomitantly to strategies for their recombinant overexpression in suitable hosts, several approaches have been adopted along with optimization of immobilization processes to achieve affordable and reusable enzymatic system (Di Cosimo, McAuliffe, Pouloseb, & Bohlmannb, 2013; Durán, Rosa, D'Annibale, & Gianfreda, 2002; Jesionowski, Zdarta, & Krajewska, 2014 and Mateo, Palomo, Fernandez-Lorente, Guisan, & Fernandez-Lafuente, 2007). In this work an empirical modeling technique, named Response Surface Methodology (RSM), was used to optimize laccase immobilization yield. In RSM statistically designed experimental models are carried out to evaluate the relationship between a set of controllable experimental factors and observed results, thus to identify the optimum conditions for a multivariable system. We adopted one of the most common design, the Box Behnken (Box & Behnken, 1960), that reduces the number of experimental trials for its application and resulted in high efficiency of modeling of multiple parameters and their interactions (Costa Ferreira et al., 2007).

Stability and catalytic parameters of immobilized laccase were also assessed in comparison with the soluble counterpart. The solid optimized biocatalyst was exploited in the clarification of raw orange juice investigating the effect of laccase treatment on juice phenolic composition.

2. Materials and methods

2.1. Materials

Recombinant POXA1b laccase from *P. ostreatus* expressed in the eukaryotic host *Pichia pastoris* was provided by Biopox srl (Italy). Epoxy activated poly(methacrylate) beads (Sepabeads EC-HFA) were purchased from Resindion srl (Italy). All reagents were purchased from Sigma–Aldrich Corp. (St. Louis, MO) unless otherwise specified.

2.2. Fruit juice extraction

Mature ripened orange (*Citrus sinensis*), pomegranate (*Punica granatum*), apricot (*Prunus armeniaca*), peach (*Prunus persica*), cherry (*Prunus avium*) and apple (*Malus domestica*) fruits were obtained from a major market in Naples, Italy. The oranges were washed and peeled. The juice was extracted using a domestic juice extractor. The pomegranate fruit was peeled and the skin covering seeds was removed. The remaining part were homogenized by blender (Waring, USA) and centrifuged at 10,200×*g* for 5 min. The pellet was discarded. The apricot, peach and cherry were pitted and homogenized by Waring blender. The apple was peeled and homogenized. All the juices were immediately processed after the extraction.

2.3. Enzyme immobilization

Immobilization reaction was performed incubating a variable quantity of poly(methacrylate) beads with 30 mL of 20,000 U L^{-1} rPOXA1b laccase solution (1000 U g^{-1} of protein) in $5 * 10^{-2}$ M of different buffers (sodium citrate for pH 3, sodium phosphate for pH 6, Tris–HCl for pH 9) under magnetic stirrer (200 rpm) for 1 h at room temperature. Afterwards the supernatant was decanted and the biocatalyst particles washed with 30 mL of $5 * 10^{-2}$ M phosphate buffer for 5 min for 3 times. Laccase activity was measured in the rinsing solution to calculate the immobilization yield (Y) as ratio between the total enzymatic IU presents in solution before (Ui) and after (Uf) immobilization reaction ($\%Y = [1 - (Uf/Ui)] * 100$). Laccase activity (IU) was assayed by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (see enzyme assay, Section 2.5). The biocatalyst was stored at 4 °C in the phosphate buffer with $5 * 10^{-3}$ M glycine to saturate possible not reacted sites.

2.4. Experimental design and statistical analysis

The RSM was set up through a Box Behnken design choosing three independent variables (quantity of beads, pH and temperature) selected on the basis of preliminary experiments and the related experimental domain was fixed for each variable (Table 1). The ratio between the amount of enzyme and beads was varied keeping constant the volume and concentration of laccase solution and varying the quantity of beads (expressed in grams). A total of 15 or more experimental sets were carried out for each run.

Data obtained through the experimental matrix were computed for the determinations of regression coefficient of the second order multiple regression model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x^2 + \sum_{j=1}^{k} \sum_{i=1}^{j-1} \beta_{ij} x_i x_j$$

where Y is the predicted response variable, β_0, β_i, β_{ii} and β_{ij} are regression coefficient of the model, x_i, x_j represent the independent variables in the form of actual value. The analysis of regression and

Table 1
Experimental Box Behnken design on the selected independent factors. For each factor three values (levels) were selected: high, low and average level.

	g of beads[a]	pH[b]	Temperature[c] (°c)
1	Average	Average	Average
2	High	Average	High
3	Low	Low	Average
4	Average	High	Low
5	Low	Average	Low
6	Average	Low	Low
7	Average	High	High
8	High	Average	Low
9	Average	Average	Average
10	Low	High	Average
11	Low	Average	High
12	Average	Average	Average
13	Average	Low	High
14	High	High	Average
15	High	Low	Average

[a] Selected levels for g of beads are 1, 4.5, 8 for low, average, and high, respectively.
[b] Selected levels for pH are 3, 6, 9 for low, average, and high, respectively.
[c] Selected levels for temperature are 4, 22, 40 °C for low, average, and high, respectively.

variance was performed by Minitab 16 (Minitab Inc., LEAD Technologies).

2.5. Enzyme assay

Laccase activity was assayed at 25 °C by monitoring the oxidation of ABTS at 420 nm ($\varepsilon_{420} = 36 \times 10^3\ M^{-1}\ cm^{-1}$). The assay mixture contained $2 * 10^{-3}$ M ABTS in $1 * 10^{-1}$ M sodium citrate buffer, pH 3.0. Laccase activity towards 2,6-dimethoxyphenol (DMP) was assayed in a mixture containing $1 * 10^{-3}$ M DMP in McIlvaine's citrate phosphate buffer adjusted to pH 5.0. Oxidation of DMP was followed by an absorbance increase at 477 nm ($\varepsilon_{477} = 14.8 \times 10^3\ M^{-1}\ cm^{-1}$).

Immobilized enzyme activity was assayed incubating 1 mg of beads in 1 mL of substrate in the corresponding reaction buffer. The activity was determined by measuring, every 30 s, change in absorbance and following the reaction for 2 min. Enzymatic units were expressed as U g^{-1} of beads.

2.6. Characterization of free and immobilized laccase

2.6.1. Topographical characterization

Scanning electron microscope (SEM) analysis of epoxy activated poly(methacrylate) beads, before and after the laccase grafting step, were achieved using SEM Ultra Plus (Zeiss, Germany) with FEG (field emission gun) source operated at 10 kV. SEM samples were sputtered with gold through Sputter coater HR 208 (Cressington, England), achieving a gold layer thickness of 20 nm.

2.6.2. Determination of kinetic parameters

Michaelis–Menten constants K_M values were estimated for free and immobilized laccases using the software GraphPad Prism (GraphPad Software, USA; http://www .graphpad.com/) on a wide range of substrate concentrations ($5 * 10^{-5} - 3 * 10^{-3}$ M) trough the following equation:

$$V = \frac{V_{max}[S]}{K_M[S]}$$

where V is the velocity of the reaction, V_{max} is the maximum velocity of the reaction and [S] is the concentration of the substrate.

Enzyme activity was expressed in international units (IU).

2.6.3. Effect of pH and temperature

Laccase activity (free or immobilized) as a function of pH was assayed using ABTS and DMP as substrates in McIlvaine buffers (pH 2.0–8.0) at room temperature. The effect of temperature on laccase activity towards ABTS was evaluated in the temperature range of 25–85 °C in $5 * 10^{-2}$ M sodium phosphate buffer adjusted to pH 6.0. The activity was assayed as previously described.

2.6.4. Stability at pH and temperature

pH stability was evaluated by incubating enzymes in $5 * 10^{-2}$ M citrate buffer pH3, $5 * 10^{-2}$ M phosphate buffer pH 6 and $5 * 10^{-2}$ M Tris–HCl pH 9 at 25 °C up to 65 days. Thermal stability was determined incubating free and immobilized laccases at the selected temperatures (25 °C, 55 °C, 65 °C) in $5 * 10^{-2}$ M phosphate buffer pH 6 until day 40. Residual laccase activity was assayed at room temperature in standard conditions with ABTS. The half-life (t1/2) at different temperatures or pH is referred to the time corresponding to the 50% of residual activity. This value was extrapolated from the tendency curve related of enzyme deactivation in each condition.

2.6.5. Storage stability

For testing the storage stability of enzymes, free and immobilized laccase in $5 * 10^{-2}$ M sodium phosphate buffer pH 6.5 were stored at 4 °C for 6 months. Remaining laccase activity was assayed at room temperature in reference conditions at different times.

2.6.6. Reusability

Several consecutive oxidative cycles were performed in standard condition. At the end of each oxidation cycle, the immobilized laccase was washed three times with $5 * 10^{-2}$ M phosphate buffer pH 6.5 for 5 min under magnetic stirrer (200 rpm) and gravity filtered on a gauze. The procedure was then repeated with a fresh aliquot of substrate.

2.7. Fruit juices treatment

4 ml of fresh fruits juice were incubated with 0.5 g of immobilized enzymes (2000 U g^{-1}) for 1 h at room temperature in continuous stirring. The juices were then decanted and analyzed for phenol content. Controls were performed incubating 0.5 g of epoxy activated poly(methacrylate) beads in 4 ml of fruit juice in the same conditions.

Ultrafiltered samples were obtained treating 4 ml of fruit juice on 10,000 Dalton cut-off membrane on an amicon device (Merck Millipore Corporation, Italy).

2.8. Extraction and determination of total phenolic compounds

The extraction procedure was carried out with 0.5 mL juice/methanol (1:1) for 30 min at −20 °C. The sample was centrifuged for 30 min at 23,900×g and the supernatant recovered was assayed.

The total phenolic content of fruit was determined by using Folin–Ciocalteu assay (Singleton & Rossi, 1965). Gallic acid stock solution, (1 mg mL^{-1}) and standard concentrations of 0, 10, 25, 50, 100, 250 and 500 μg mL^{-1} were prepared in deionized water. The Folin–Ciocalteu procedure consisted of transferring 50 μL standard or sample into a 4–5 mL borosilicate tube, followed by additions of 430 μL H_2O and 20 μL Folin–Ciocalteu reagent. After mixing the samples, 50 μL 20% sodium carbonate and 450 μL H_2O were added. The sample mixtures were allowed to stand for 1 h at room temperature. The absorbance was measured at 725 nm. The phenolic content of samples was measured against the gallic acid (GA) calibration standard (0–500 ppm). Phenol reduction was estimated as percentage respect to untreated samples.

2.9. Mass spectrometry analyses

In order to obtain a molecular investigation of species occurring in the different samples, aliquots of methanolic extracts from fruit juice were submitted to mass spectral analyses by using both GCMS and LCMS/MS techniques.

2.9.1. GC–MS analysis

Aliquots of 1 μL of each methanolic extracts were directly analyzed using a GC–MS equipped with a 5975 MSD quadrupole mass spectrometer (Agilent technologies, USA) and a gas chromatograph 7820A (Agilent technologies, USA) by using a ZB-5MS fused silica capillary column (30 m length, 0.25 mm ID, 0.25 μm Film Thickness) from Phenomenex, (USA). The injection temperature was 250 °C. During analyses, the oven temperature was increased from 60 °C to 300 °C at 10 °C min^{-1} and held at 300 °C for 10 min. Electron Ionization mass spectra were recorded by continuous quadrupole scanning at 70 eV ionization energy, in the mass range 50–600 m/z. Each specie was interpreted on the basis of electron impact spectra (NIST 2011 library, Scientific Instrument Services, USA, and Analyst Software, SCIEX, Canada).

2.9.2. LC–MS/MS analysis

Methanolic extracts were dried under vacuum and resuspended in 200 μL of 0.1% formic acid. The resulting samples were filtrated using cellulose acetate spin filters (0.22 μm) from Agilent and 1 μL of each sample was analyzed by nanoLC Chip MS/MS, using a CHIP MS 6520 QTOF equipped with a capillary 1200 HPLC system and a chip cube (Agilent Technologies, USA). After loading, the samples were first concentrated and washed at 4 μL min^{-1} in 40 nL enrichment column (Agilent Technologies chip, USA), with 0.1% formic acid as eluent, and then fractionated on a C18 reverse-phase capillary column (75 μm × 43 mm in the Agilent Technologies chip, USA) at flow rate of 400 nL/min with a linear gradient of eluent B (0.1% formic acid in 95% ACN) in A (0.1% formic acid in 2% ACN) from 7% to 60% in 50 min. Mono charged analytes were selected and analyzed using data-dependent acquisition of one MS scan (mass range from 100 to 1500 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Collision energy (CE) applied during fragmentation is calculated by the sequent empirical equations: CE = 4V/100 Da − 2.4V. Raw data from nanoLC–MS/MS were analyzed using Qualitative Analysis software (Agilent MassHunter Workstation Software, version B.02.00, USA).

2.10. Statistical analysis

Those analysis not concerning RSM represent the mean of triplicate measurements and are expressed as mean ± S.D. (standard deviation of the mean). One-way analysis of variance (ANOVA) followed by the unpaired Student's t test or Tukey's test was used. $p < 0.05$ was considered statistically significant, unless otherwise specified.

3. Results and discussions

3.1. Immobilization of laccase on Sepabeads EC-HFA

Applicability at industrial scale of an enzyme is dependent on its performances as well as on its manufacturing costs, which have to be conformed to commercial and industrial demands. In this context, design of suitable immobilization process and its optimization in operating conditions are of utter importance. In the present work covalent immobilization of rPOXA1b laccase on epoxy activated poly(methacrylate) beads was chosen as a promising method to provide a useful catalytic system for several applications (Mateo et al., 2007). A RSM approach was applied to define process parameters improving laccase immobilization through the characteristic three major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. Box Behnken design was performed selecting the range of levels variation for each factor on the basis of empirical and theoretical consideration concerning enzyme stability and volumetric constrain. As a fact, a pH range from 3 to 9 was chosen, avoiding extreme pH values that could negatively affect enzyme stability (Miele et al., 2010). Moreover, the quantity of poly (methacrylate) beads usable for this process is limited by the necessity of incubating them in an enzymatic solution properly covering their surface. Laccase immobilization yield was considered as response variable and it was evaluated as ratio between the total enzymatic IU presents in solution before and after immobilization reaction (%Y = [1 − (Uf/Ui)] * 100). According to this design, 15 runs, replicated three times, were performed and experimental data (Table 2 column named "Yield") were obtained. Results obtained after running the trials of the Box Behnken design were fitted to a factorial equation to explain the dependence of laccase immobilization yield on the designed variables as shown in the equation:

$$Y = -64.1346 - 0.958050 * \text{g of beads} + 30.7526 * \text{pH} - 2.17130 * \text{pH} * \text{pH} + 1.07143 * \text{g of beads} * \text{pH}$$

The second-order regression equation provided the levels of immobilized laccase as a function of parameters which can be presented in terms of coded factors. The factors can affect the results in different ways and are integrated in the equation as "linear" (g of beads * pH), "square" (pH * pH) or "interacting" (g of beads * pH) coefficients. The statistical significance of regression equation was checked by *F*-test, and the analysis of variance (ANOVA) is shown in Table 2B.

The Model *F*-value found of 8.21 corresponding to *p*-value 0.016 implies that the model is significant. The value of the determination coefficient R^2 calculated from the quadratic regression model

Table 2
(A) experimental conditions of the experimental design for laccase immobilization and the corresponding experimental responses. (B) Analysis of variance (ANOVA) for the fitted quadratic polynomial model of laccase immobilization.

	g of beads	pH	Temperature	Yield (%)
(A)				
1	4.5	6	22	70
2	8	6	40	74
3	1	3	22	13
4	4.5	9	4	70
5	1	6	4	45
6	4.5	3	4	24
7	4.5	9	40	50
8	8	6	4	94
9	4.5	6	22	70
10	1	9	22	45
11	1	6	40	36
12	4.5	6	22	47
13	4.5	3	40	23
14	8	9	22	97
15	8	3	22	20

Source	DF	Seq Ss	Adj SS	Adj MS	*F* value	Prob > F
(B)						
Regression model	9	9177.07	9177.07	1019.67	8.21	0.016
Linear	3	7117.50	7117.50	2372.50	19.11	0.004
g of beads enzime/beads	1	2664.50	2664.50	2664.50	21.46	0.006
pH	1	4140.50	4140.50	4140.50	33.36	0.002
Temperature	1	312.50	312.50	312.50	2.52	0.173
Square	3	1432.82	1432.82	477.61	3.85	0.091
g of beads enzime/beads * g of beads enzime/beads	1	22.02	3.39	3.39	0.03	0.875
pH * pH	1	1406.79	1410.01	1410.01	11.36	0.020
Temperature * Temperature	1	4.01	4.01	4.01	0.03	0.864
Interaction	3	626.75	626.75	208.92	1.68	0.285
g of beads enzime/beads * pH	1	506.25	506.25	506.25	4.08	0.099
g of beads enzime/beads * Temperature	1	30.25	30.25	30.25	0.24	0.642
pH * Temperature	1	90.25	90.25	90.25	0.73	0.433
Residual	5	620.67	620.67	124.13		
Lack of fit	3	268.00	268.00	89.33	0.51	0.716
Pure error	2	352.67	352.67	176.33		
Total	14	9797.73				
R^2 = 93.67%	R^2 adj. = 82.26%					

DF – (Total degrees of freedom) are the amount of information data estimates.
Sequential SS (Sequential Sum of Squares) are the numerators of the linear F-statistic.
Adjusted SS – (Adjusted sums of squares) are measures of variation for different components of the model. The order of the predictors in the model does not affect the calculation of the adjusted sum of squares.
Adjusted MS – (Adjusted mean squares) measure how much variation a term or a model explains, assuming that all other terms are in the model, regardless of the order they were entered.
F value – probability distribution value used in the ANOVA.

was 0.9367, while the value of the adjusted R^2 was 0.8226 indicating high degree of correlation between the observed and predicted values. Thus, the regression model provides a good explanation of the relationship between independent variables and the response (Yield). Other values reported in the ANOVA Table 2B are used by the software to calculate the *p*-value for a term and the R^2 statistic. Usually, interpretation of results is based on the *p*-values, the R^2 statistic and the adjusted R^2 statistic instead of the degrees of freedom, the sums of squares and the adjusted mean squares.

The lack-of-fit test measures the failure of the model to represent data in the experimental domain, at points which are not included in the regression. This test is desired to be non-significant to support the model (Montgomery, 2001). In this study, the lack of fit *F*-value of 0.51 with *p*-value 0.716 implies that the lack of fit is not significant.

All these results suggest that the quadratic model is statistically significant for the response, and therefore it can be used for further analysis. The 3D response surface plots (Fig. 1) were obtained by plotting the response (yield) on the *Z*-axis against any two variables while keeping the third variable at its average level. Based on the above analysis, in the range of our research, high pH values and high quantity of enzyme positively affects laccase immobilization, while temperature seems not to affect immobilization yield in the range of the tested conditions.

The maximum laccase immobilization yield of 100% was predicted at the following optimum conditions: 8 g of beads for 30 ml of 20,000 U L^{-1} rPOXA1b solution, in a buffer at pH 9, incubating at 4 °C. Another three experiment sets were carried out to confirm the prediction. An average of 98 ± 5% yield was achieved, theoretically corresponding to an immobilization of 75 U g^{-1} of beads, thus indicating an excellent fit with the predicted value. Notably, we reached 2000 ± 100 U g^{-1} of beads with a remarkable increase of enzyme specific activity respect to that of the free counterpart of more than 25 folds. This effect has been already reported in other works (Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernández-Lafuente, 2013), and it is probably due to 3D structure variations after covalent attachment on a solid support. Thus, the process could facilitate a conformational rearrangement of the active site, *i.e.* unveiling the binding pocket, that positively affects the catalytic proprieties.

As expected, when the immobilization process was performed at room temperature, similar yields (96%) were obtained, thus providing the conditions for a less expensive procedure.

3.2. Immobilized enzymatic system characterization

3.2.1. Morphological characterization

Morphologies of beads surface were investigated using Scanning Electron Microscope before and after immobilization procedure. As shown in SEM micrographs (Fig. 2B), surface of the treated beads was looked like a mesh, and showed very compact structures, revealing an increase of surface roughness upon enzyme loading respect to the not-treated ones (Fig. 2A).

3.2.2. Kinetic analysis

Apparent K_M constants were determined for the immobilized enzyme against two different laccase substrates, ABTS and DMP, and compared with the free counterpart (Supplementary material, Fig. S1). An opposite effect was observed for the two tested substrates. K_M value *vs* ABTS of the immobilized enzyme (0.032 ± 0.006 mmol l^{-1}) is lower (*p*-value < 0.05) in respect to that observed for the soluble rPOXA1b (0.063 ± 0.004 mmol l^{-1}). Conversely, a slight increase (*p*-value < 0.05) in the K_M value *vs* DMP was observed for the solid catalyst (0.227 ± 0.030 mmol l^{-1}) respect to the free enzyme (0.160 ± 0.010 mmol l^{-1}). Expected phenomena occurring during enzyme immobilization —such as electrostatic and partitioning effects in the immobilized enzyme microenvironments, substrate mass transfer effects and/or changes in enzyme conformation— may differently affect properties of immobilized catalyst in a substrate dependent manner (steric hindrance, reaction mechanism *etc.*) (Pezzella et al., 2014).

3.2.3. Temperature and pH activity profiles

The effect of the temperature on the activity of free and immobilized laccases was determined in the range 25–85 °C. Both catalysts display a maximum at 75 °C, although free rPOXA1b retains most of its activity in a wider temperature range when compared with the immobilized enzyme (Supplementary material, Fig. S2). A reduced flexibility, caused for example by the occurrence of multi-point attachments, may explain the observed results.

Enzyme immobilization determines a shift in the optimal pH (from 4 to 5) towards ABTS along with a reduced enzyme activity at more acidic pHs Supplementary material, Fig. S3. On the other hand, free and immobilized laccase show a similar pH activity profile against DMP.

3.2.4. Thermostability and pH stability

Enzyme thermostability was evaluated incubating free and immobilized rPOXA1b at selected temperatures (Supplementary material, Fig. S4). The immobilized enzyme displays and increased stability at all tested temperatures with a twofold increase in the $t_{1/2}$ values (Table 3).pH stability was investigated at values 3, 6 and 9 (Table 3 and Supplementary material, Fig. S5). The solid catalyst exhibits an enhanced stability at all pH values (*p* value < 0.05, for each value of immobilized *vs* free enzyme), further boosting rPOXA1b peculiar stability at alkaline pH (Miele et al., 2010) and improving its performances at acidic pH.

3.2.5. Immobilized biocatalyst stability

Laccase immobilized on Sepabeads was efficiently stored at 4 °C in 5 * 10^{-2} M phosphate buffer pH 6.5 preserving almost 95% if its activity after 4 months and about 90% after 6 months. Under the same storage conditions, soluble laccase shows a 50% drop in activity after six months.

As far as enzyme reusability, immobilized enzyme retains 67% of initial activity after ten cycles of ABTS oxidation at room temperature (Supplementary material, Fig. S6). These performances, having a great influence on process economics, are superior than those previously reported, showing 50% residual activity under similar conditions (Liu et al., 2012 and Rekuć, Bryjak, Szymańska, & Jazębski, 2009).

3.3. Fruit juice treatment with immobilized laccase

The effectiveness of immobilized laccase in fruit juices treatment was investigated choosing orange juice as a model. Raw orange juice was incubated for 1 h at room temperature with the solid catalyst and the total phenol content analyzed in comparison with that of an ultra-filtered sample. Ultra-filtered juices are not always stable, but rather tend to produce pronounced subsequent haze, caused by reactive phenolics compounds that cannot be retained by the ultra-filtration membrane (Neifar et al., 2011). Laccase treatment generates up to 45% reduction, whereas after ultrafiltration, only a 15% phenols decrement was measured. No phenol reduction was observed in the control tests (fruit juice incubated with the poly(methacrylate) solid support without enzyme) . Thus, it is possible to rule out a possible effect due to endogenous polyphenol oxidase activities.

The solid catalyst can be reused up to three times in juice treatment without losing efficiency.

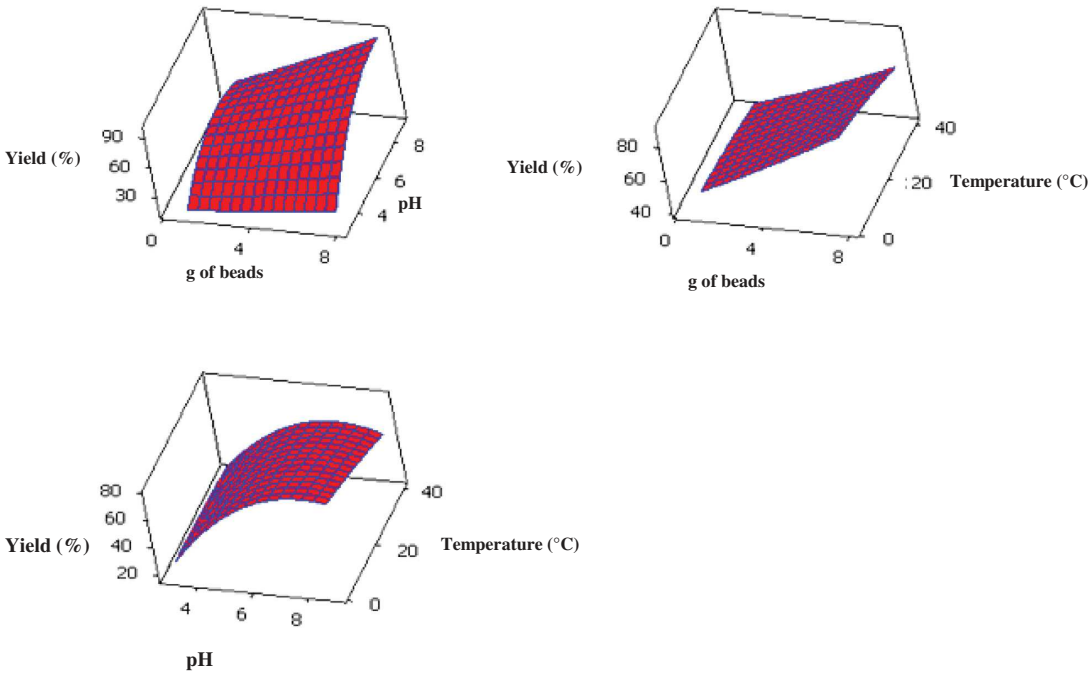

Fig. 1. 3D surface plot for the laccase yield immobilization as a function of temperature, pH and enzyme/beads ratio.

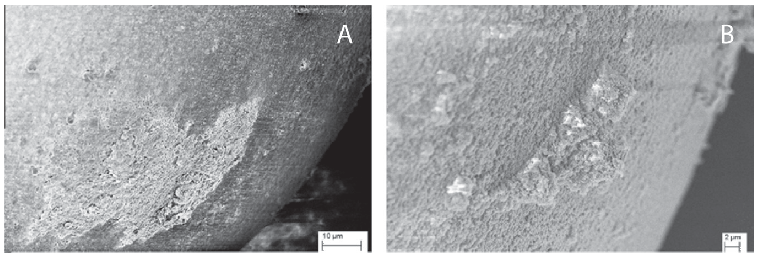

Fig. 2. Sem image analysis of epoxy activated poly(methacrylate) beads before, (A) and after (B) laccase immobilization.

Table 3
Temperature and pH stability profiles of (A) free and (B) immobilized laccase expressed as half-life. ABTS was used as the substrate for the enzyme assay. Each value represents the mean of triplicate measurements and varies from the mean by not more than 10%.

	$t_{1/2}$ temperature (h)			$t_{1/2}$ pH (day)		
	25 °C	55 °C	65 °C	pH 3	pH 6	pH 9
Free rPOXA1b	7.1	3.5	0.8	2.9	6.8	23.1
Immobilized rPOXA1b	16.3	7.8	1.5	5.7	16.0	63.7

3.3.1. Compounds identification

Citrus phenolics have been subject of increased interest in the last few years because their presence contributes to the sensory quality of fruit and juice, affecting color, bitterness, astringency, antioxidant activity and flavor (Sousa, da Rocha, Cardoso, Silva, & Zanoni, 2004). Sample aliquots of treated and not-treated raw orange juice were analyzed by LC–MSMS mass spectrometry to evaluate flavanones content. The total ion current chromatograms (TIC) showed a similar behavior. No decrease in flavanones contents could be appreciated in the LC–MSMS analyses (Supplementary material, Fig. S7). Thus, laccase treatment does not affect these health-effective molecules (Silva et al., 2014), probably due to a peculiar affinity towards other species present in the orange juice, such as phenols, that could compete for or avoid the oxidation of flavanones. This finding is unexpected if compared to the natural or induced oxidation process occurring in other fruit beverages like grape juice and wine, where the flavanones oxidation plays a prominent role in the formation of brown pigments (Shahidi & Naczk, 2003). Moreover, mass spectrometry analyses were used to evaluate the laccase treatment effect on phenolic content. Phenol standard mixture, containing coumaric acid, caffeic acid, synapinic acid, ferulic acid, vanillic acid and syringic acid (Barberis et al., 2014) was analyzed by GC–MS in order to set up the best chromatographic and mass spectral conditions (Supplementary material, Fig. S8). Mass spectral analyses were carried out on laccase treated orange juices. Commercial orange juice was used as control. The species were identified on the basis of electron impact mass spectra showing a general decrease in phenol content after laccase treatment. In particular, while caffeic acid seems to be unaffected by laccase treatment, the TIC showed the

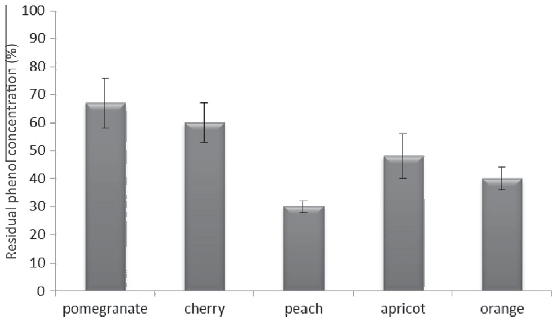

Fig. 3. The reduction effect of phenol content in different fruit juices after immobilized laccase incubation. The values are expressed in percentage of residual phenol concentration. Standard deviations derive from three independent replicates.

decrease of coumaric and ferulic acid. Moreover, in the laccase treated juice, the chromatograms showed an intense reduction of vinylguaiacol, a degradation product of ferulic acid. Vinylguaiacol is described as possessing a peppery/spicy aroma and is considered a potent off-flavor (Naim, Striem, Kanner, & Peleg, 1988). Thus, it can be conveyed that laccase treatment improves orange juice sensory profile and extends its shelf life.

Furthermore, in order to evaluate the versatility of the developed catalytic system, immobilized laccases were tested on different raw fruit juices. As reported in Fig. 3 the immobilized laccase system is able to sensitively reduce the phenol content of several fruit juices at a level comparable with that achieved for the orange juice.

4. Conclusion

Optimal conditions for laccase immobilization were set-up through a RSM approach using the Box Behnken design. Laccase based juice treatment allowed to reach up to 45% phenol reduction. The health-effective flavanones molecules are not affected by the treatment. Furthermore, laccase treated juice displays an improved sensory profile, due to the reduction of vinyl guaiacol, a potent off-flavor possessing a peppery/spicy aroma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.074.

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CHAPTER 5

Laccase assisted synthesis of "green" dyes

5. General introduction

Dyes are defined as substances that, when applied to a substrate, provide color by a process that alters, at least temporarily, any crystal structure of the colored substances (Kirk-Othmer 2004, Bafana *et al.,* 2011). Such substances with considerable coloring capacity are widely employed in the textile, pharmaceutical, food, cosmetics, plastics, photographic and paper industries (Zollinger 1987, Carneiro *et al,* 2007). The dyes can adhere to compatible surfaces by solution, by forming covalent bond or complexes with salts or metals, by physical adsorption or by mechanical retention (Kirk-Othmer 2004, Bafana *et al.,* 2011). Dyes are classified,according to their application and chemical structure, and they are composed of a group of atoms known as chromophores, responsible for the dye color. These chromophore-containing centers are based on diverse functional groups, such as azo, anthraquinone, methine, nitro, arilmethane, carbonyl and others. In addition, electrons withdrawing or donating substituents so as to generate or intensify the color of the chromophores are denominated as auxochromes. The most common auxochromes are amine, carboxyl, sulfonate and hydroxyl (Christie 2001, Dos Santos *et al.,*2007, Arun Prasad *et al.,* 2010). It is estimated that over 10,000 different dyes and pigments are used industrially and over 7 x 10^5 tons of synthetic polymeric dyes are annually worldwide produced (Ogugbue *et al.,* 2011). Among the industries, up to 200,000 tons of these dyes are lost to effluents every year during the dyeing and finishing operations, due to the inefficiency of the dyeing process (Ogugbue *et al.,* 2011). Nowadays, there is an increasing demand on the chemical industry to develop eco-friendly processes with the use of biocatalysts that represent an attractive route towards one-step safe synthesis. For this reason, in the last decades, bio-based alternatives with the least environmental impact have been widely researched for dye synthesis (Pezzella *et al.,* 2015). In this context, enzymatic processes have been extensively exploited, and laccases represent one of the most promising tools.

In this chapter the fungal laccase POXA1b was applied to the synthesis of two phenolic/amino dyes with two different application: a resorcinol/2,5 dabsa coupling for the textile industry (Section 5.1), and α-naphtol/2,5 dabsa coupling as an alternative protein staining agent (Section 5.2).

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Green routes towards industrial textile dyeing: A laccase based approach

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ABSTRACT

Laccase-catalyzed synthesis of dye molecules represents a green choice to reduce the environmental footprint of conventional synthetic processes. Textile industry will benefit from this green technology since the synthesized dyes can be exploited to colour different fabrics.

This work describes the application of the *Pleurotus ostreatus* POXA1b laccase in polymeric dye synthesis using resorcinol and 2,5-diaminobenzenesulfonic acid (2,5-DABSA) as substrates. The potential of the resorcinol/2,5-DABSA coupling route was transferred to a chemical industry, Setaş Colour Center, by introducing a greener synthesis step within the process routinely used for textile dyeing. Dye synthesis was performed at different precursor ratios (1:1 and 1:10 2,5-DABSA: resorcinol) and their dyeing properties were compared on different fibres. The two mixtures of synthesized dyes proved to be effective on nylon and wool, with 1:10 ratio displaying the best performances in terms of dyeing efficiency and colour strength. Good and comparable end quality and "performances during use" were observed for nylon and wool coloured with both synthesized dyes.

1. Introduction

Implementation of biotechnology in the chemical synthesis aims at reducing the environmental footprint of conventional synthetic processes. Green catalytic alternatives are particularly needed in the production of dyes considering their huge global production and the harsh operative conditions required for their synthesis. For this reason, in the last decades, bio-based alternatives with the least environmental impact have been widely researched for dye synthesis looking for safe oxidant, such as oxygen and green catalyst, such as enzymes [1–4]. In this context, enzymatic processes exploiting laccases represent one of the most promising tools. Laccases (EC 1.10.3.2) are well-known multicopper phenol oxidases able to oxidize a wide spectrum of both phenolic and non-phenolic substrates to radical forms [5,6]. The oxidation of substrates by laccases takes place under mild conditions, using oxygen as co-substrate and producing water as by-product [7–10]. In particular, fungal laccases display a wider range of oxidizable substrates in comparison with bacterial ones, due to the high redox potential of their T1 copper. In addition, if these enzymes are secreted in the extracellular medium, their recovery becomes more easy and cost-effective. As a fact, taking advantage of their ability to catalyse homo- or hetero-molecular coupling of reactive intermediates, fungal laccases have been applied to the synthesis of polymeric dyes for textile and hair dyeing [11–14].

In this work a high redox potential (HRP) fungal laccase has been exploited in a biosynthetic pathway to synthesize polymeric phenolic/amino dyes based on 2,5-diaminobenzenesulfonic acid (2,5-DABSA) and resorcinol. The former has been already successfully used for the *in situ* colouration of both cotton and wool fibres, mainly coupled to catechol as dye modifier [11,12,14,15]. Oxidative coupling is the main mechanism behind this laccase catalyzed reaction, leading to C—N hetero-coupling between the two substrates on a backbone of catechol polymer resulted from a C—C homo-coupling [12,14,16]. On the other hand, resorcinol has been barely exploited in fibre dyeing. This molecule, unlike catechol, can be synthesized by a selective reaction route limiting the production of harmful by-products [17–20].

Dye synthesis was performed at alkaline pH to promote both C—N cross-coupling [14] and resorcinol polymerization. Furthermore, high pH and temperature (pH 8 and 60 °C) were applied to increase substrates solubility in order to potentially obtain the

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highest yield of coloured products for the industrial dye bath. In this frame, the use of an enzyme able to cope with high pH and temperature is mandatory.

The *P. ostreatus* laccase POXA1b displays a well-known activity and stability at the required conditions [21], addressing the reaction constraints.

The potential of the resorcinol/2,5-DABSA coupling route was transferred to a chemical industry, Setaş Colour Center, and enzymatically synthesized dyes were straight applied in their existing textile dyeing procedure. A comparison between enzymatic and chemical dyes was performed in terms of their dyeing effectiveness and fastness properties.

2. Materials and methods

2.1. Materials

Recombinant POXA1b laccase from *P. ostreatus* [22], expressed in the yeast *Pichia pastoris* under the control of *AOX1 promoter* was produced and kindly provided by Biopolis S.L. (Spain) in the frame of INDOX European Project.

All reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

2.2. Laccase activity

Laccase activity was determined using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) as substrate following the reaction at 420 nm ($\varepsilon_{420} = 36 \times 10^3\ M^{-1}\ cm^{-1}$), as previously reported [23].

Determination of laccase activity towards 2,5-DABSA (1–25 mM) and resorcinol (10–50 mM) was performed by estimating the increase in absorbance at 460 and 490 nm after an incubation of 5 and 10 min at 60 °C, respectively. Assay mixture contained 1 U mL^{-1} of POXA1b laccase (estimated using ABTS as substrate) in 50 mM Tris HCl, pH 8.

2.3. Dye synthesis

Reactions were carried out at different ratios between the two precursors in 50 mM Tris HCl, pH 8.0 at 60 °C under agitation (150 rpm) for 6 h using 1 U mL^{-1} (assayed towards ABTS) of POXA1b. Two ratios 2,5-DABSA: resorcinol were tested (1:1, 1:10), using 10, 25 or 50 mM resorcinol and the corresponding concentrations of 2,5-DABSA. When 50 mM resorcinol was used, the 1:1 ratio was not performed because of 2,5-DABSA limit of solubility in the conditions used (25 mM). Reactions were followed by hourly registering the absorbance spectra (380–800 nm), and subtracting spectra of substrates in the absence of enzyme as blank (Jasco V-530 UV/vis).

The two ratios 2,5-DABSA: resorcinol were scaled-up to 1L using 25 mM 2,5-DABSA and 25 mM resorcinol for the 1:1 reaction, and 25 mM 2,5-DABSA and 250 mM resorcinol for the 1:10. At the end of the reactions, products were analysed using a A-8453 UV–vis Spectrophotometer (A-61103)- Agilent system. Synthesized dyes were concentrated by nano-filtration equipment (handmade equipment by Setaş) using a polyamide membrane (AFC40, PCI membrane) to a final volume of 250 mL.

2.4. Multifibre test

The concentrated dyes were used for the multifibre dyeing (acetate, cotton, nylon, polyester, acrylic, wool). 5 g of each textile were dyed in 100 mL water containing 0.2 g L^{-1} Setacid VS-N, 10 g L^{-1} Setalan PM71, 20 g L^{-1} Na_2CO_3 using 8% v/v of 1:1, 1:10 ratio and Nyloset Brown N2R (reference dye). Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine (Copower Technology, LTD, Taipei Taiwan).

2.5. Fibre dyeing

Nylon fibres (5 g textile) were dyed using different concentrations (2 ÷ 8% v/v) of each concentrated dye (1:1, 1:10 ratio and Nyloset Brown N2R), in 100 mL water containing 0.2 g L^{-1} Setacid VS-N, 10 g L^{-1} Setalan PM71, 20 g L^{-1} Na_2CO_3. Dyeing was performed for 1 h at 102 °C in an IR Dyeing Machine (Copower Technology, LTD, Taipei Taiwan).

After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath).

Wool fiber (5 g textile) were dyed using different concentrations (2 ÷ 8% v/v) of each concentrated dye (1:1, 1:10 ratio and Nyloset Brown N2R), in 100 mL water bath containing 0.5 g L^{-1} Setalan PM 71, 3.0 g L^{-1} Ammonium Sulfate, 2 g L^{-1} Setacid VS-N, 0.2 g L^{-1} Na_2CO_3. Dyeing was performed for 1 h at 98 °C. After the first dyeing procedure, the same bath was used to dye another 5 g textile piece (second bath).

2.6. Fibre characterization

The CIELAB coordinates (L*, a*, b*) and colour strength of the samples (K/S) were evaluated using a reflectance measuring apparatus Datacolor SF600 plus. The Kubelka-Munk relationship (K/S), where K is an adsorption coefficient and S is a scattering coefficient, was applied to textiles under the assumption that light scattering is due to the fibres, while adsorption of light is due to the colorant.

The colour fastnesses of the dyed fabrics were evaluated following established test procedures: ISO 105-B02:1994-Colour fastness to artificial light: Xenon arc fading lamp test (Blue scale 1–8); ISO 105-CO6:1998-Colour fastness to domestic and commercial washing (Grey scale 1–5); ISO/DIS 105-X12:2001-Colour fastness to rubbing (Grey scale 1–5).

3. Results

3.1. Design of the process conditions

The activity of POXA1b towards 2,5-DABSA and resorcinol precursors was first assayed, and the enzyme was found able to oxidize both substrates at all the tested concentrations (Supplementary Fig. 1). In fact, a remarkable increase of absorption intensity was measured, with peaks centred at around 490 and 460 nm for resorcinol and 2,5-DABSA, respectively.

With the aim of establishing reaction conditions for dye synthesis, two different 2,5-DABSA: resorcinol ratios (1:1, 1:10) were tested setting three increasing resorcinol concentrations (10, 25, 50 mM) (Fig. 1).

As for the 1:1 ratio (Fig. 1a), at 10 mM the overall profile of the spectra increased without the presence of any peak, and a brownish colour was produced. On the other hand, at 25 mM, a peak at around 460 nm was formed, and a darker colour was observed. At 1:10 ratio (Fig. 1b) it was possible to observe the formation of a peak at around 490 nm at all the tested concentrations and higher substrate concentrations darker the colour produced. The different results obtained varying substrate ratios may reflect the different affinity/turnover of the enzyme towards the two precursors, leading to the production of different dye mixtures. In fact, at 1:1 ratio the peak at around 460 nm can be ascribable to the major conversion of 2,5-DABSA towards that of the resorcinol (Fig. 1), while when the ratio increases in favour of resorcinol (1:10 ratio), different species with an absorption peak at around 490 nm appear. Considering the operative conditions, it is not possible to rule out the existence of

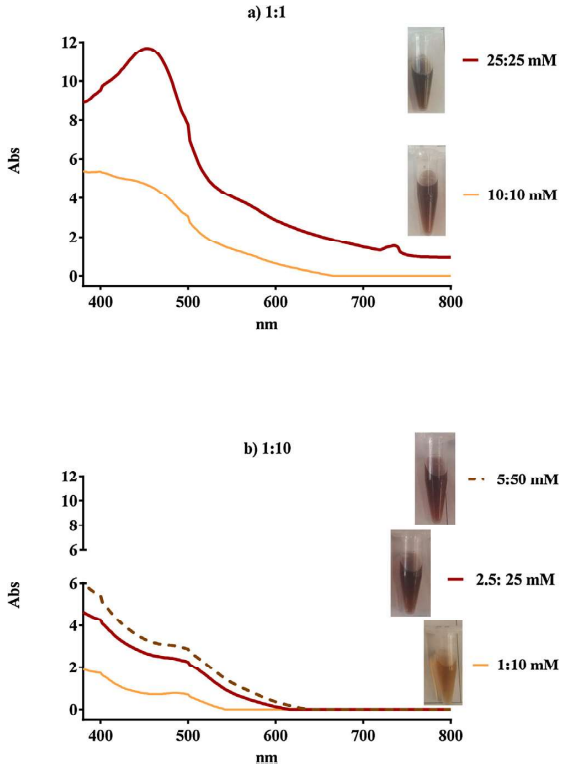

Fig. 1. Dye spectra analysis. Absorption spectra (380–800 nm) recorded after 6 h reaction with 1 U mL^{-1} of POXA1b laccase at different 2,5-DABSA/resorcinol ratios. For each ratio, different substrate concentrations have been tested. Colors corresponding to each tested ratio are shown. a) 1:1 ratio, b) 1:10 ratio. Standard deviations among three replicates were less than 5%.

spontaneous oxidation in the absence of the enzyme, as already reported for other phenols [14,24]. However, in the presence of the enzyme a significant increase in the reaction rate is observable for both reaction ratios.

Taking together all these results, both 1:1 and 1:10 ratios were used for textile dyeing with the aim to evaluate the performances of the two synthesized dyes on different fibres. A different effect is conceivable applying the two dyes.

3.2. Industrial dye synthesis

Once defined the conditions for dye synthesis, both processes were applied in conditions approaching to industrial ones. To this aim, the reactions were performed in a volume of 1 L, and concentrations of both substrates were raised up to obtain the highest yield of coloured products. Being limited by 2,5-DABSA solubility (25 mM), the reactions were carried out as follows: 25 mM 2,5-DABSA: 25 mM resorcinol (1:1 ratio, A) and 25 mM 2,5-DABSA: 250 mM resorcinol (1:10 ratio, B), as described in § 2.3.

The obtained dyes were tested in the multifiber colouration, in comparison with a commercial brown dye (Nyloset Brown N2R) from the Setaş product range. The latter was chosen according to the colour shade similarity with the new bio-dyes. According to the results reported in Fig. 2, the products of both reactions A and B showed the best dyeing performances, in terms of intensity of the colour on the fibres, on nylon and wool. A faint coloration was obtained, instead, on acetate, cotton, PES and acrylic textiles. Thus,

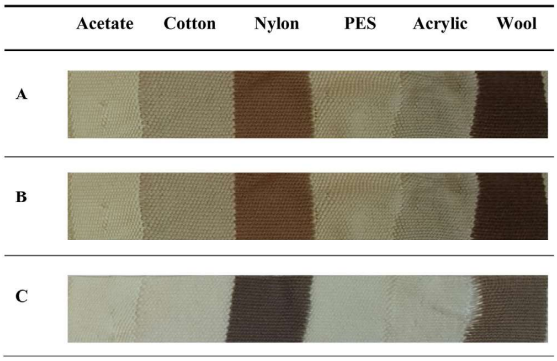

Fig. 2. Multifibre test. Swatches of different natural and synthetic fibres coloured with bio dyes synthesized from reactions A (1:1 2,5 DABSA: resorcinol), B (1:10 2,5 DABSA: resorcinol), and the reference commercial dye Nyloset Brown N2R (C).

Table 1
Properties of nylon dyed with polymeric dyes derived from reactions A (1:1 2,5 DABSA: resorcinol) and B (1:10 2,5 DABSA: resorcinol). CIELAB coordinates (L*, a*, b*) of the 8% dyed fabric for each colour are reported in the first row. Dyeing efficiency (% STR-WSUM) and colour strength (K/S) are reported for each concentration (2 ÷ 8% v/v) tested in the dyeing process. Standard deviations among three replicates were less than 5%.

Dye concentration (% v/v)	A L* 53.06; a*8.16; b*10.26		B L* 39.62; a*8.09; b*9.32	
	% STR-WSUM	K/S	% STR-WSUM	K/S
8	100	2.7	100	5.7
6	98.4	2.6	93.4	5.3
4	77.5	2.1	75.5	4.2
2	46.0	1.2	44.1	2.4

further characterization of dyeing properties of the enzymatically synthesized dyes was carried out on nylon and wool.

3.3. Characterization of dyed textiles

3.3.1. Nylon

In order to define the best dyeing conditions, nylon was coloured using different percentages (2 ÷ 8%) of nanofiltered reaction products for each dyeing bath. Table 1 reports the efficiency of the dyeing process (% STR-WSUM) for each tested concentration. These values are expressed as relative percentages respect to the dyeing process carried out using the highest dye concentration (8%). The CIELAB coordinates of the 8% dyed fabric were measured and reported in Table 1. The two different reaction ratios produced an almost similar tone, mostly different in Lightness (L*), being colour produced by the reaction B darker than A. It is noteworthy that for both conditions the efficiency of the dyeing process increased with concentration, although no significant improvement occurred using 8% in comparison with 6%. As far as the colour strength (K/S), this value increased with concentration for both reactions, as already observed for dyeing efficiency. It is remarkable that colour synthesized in reaction B displayed an higher affinity towards the fibre, in comparison to dye A. Dyeing in the remaining bath was measured for all the tested dye concentrations, since, from the industrial point of view, it is desirable to fully exploit the colour present in the dyeing bath in order to assure a constant tone on the textile for each process as well as to avoid the discharge of coloured wastewater. Fig. 3 shows that both enzymatically synthesized dyes still persisted in the remaining bath, since the textiles were coloured also after the second bath. On the other hand, no colour was present in the second bath when the commercial Nyloset N2R dye was used.

Fig. 3. Nylon dyeing efficiency in the remaining bath. a) Nylon dyed with different concentrations of dyes (2 ÷ 8% v/v) synthesized from reactions A (1:1 2,5 DABSA: resorcinol), B (1:10 2,5 DABSA: resorcinol) and the reference commercial dye Nyloset Brown N2R (C); b) nylon dyed with the remaining bath derived from each dyeing processes reported in the panel a).

Table 2
Comparison of dyeing and fastness properties of nylon coloured with Nyloset N2R (C), dye A (1:1 2,5 DABSA: resorcinol) and dye B (1:10 2,5 DABSA: resorcinol). All dye mixtures were used at 6% v/v concentration in the dyeing bath.% STR-WSUM obtained with the enzymatic dyes is expressed as relative percentage respect to the commercial dye Nyloset N2R. Standard deviations among three replicates were less than 5%.

Dye	Dyeing properties		Colour Fastness			
	% STR-WSUM	K/S	Light	Washing	Rubbing	
					Wet	Dry
C	100	2.8	4	4/5	4/5	4/5
A	68.1	2.6	2	4/5	3/4	4/5
B	156.4	5.3	2	4/5	4/5	4

According to the reported data (Table 1) the dyeing process using 6% of reaction products was further characterized also in terms of fastness properties, and results of reactions A and B were compared with those obtained with the commercial dye used in the same dyeing conditions (Table 2). Dye B displayed a better dyeing efficiency and colour strength in comparison with both dye A and the standard dye, suggesting the existence of a stronger interaction of dye B to the nylon. In fact, all fabrics dyed with enzymatically synthesized dyes showed resistance to washing and friction comparable to that of Nyloset N2R. On the other hand, nylon coloured with both A and B dyes displayed a low light fastness.

Table 3
Properties of wool dyed with polymeric dyes derived from reactions A (1:1 2,5 DABSA: resorcinol) and B (1:10 2,5 DABSA: resorcinol). CIELAB coordinates (L*, a*, b*) of the 8% dyed fabric for each colour are reported in the first row. Dyeing efficiency (% STR-WSUM) and colour strength (K/S) are reported for each concentration (2÷ 8% v/v) tested in the dyeing process. Standard deviations among three replicates were less than 5%.

Dye concentration (% v/v)	A L* 53.21; a*8.28; b*9.86		B L* 37.78; a*8.39; b*10.28	
	% STR-WSUM	K/S	% STR-WSUM	K/S
8	100	2.8	100	7.1
6	88.7	2.5	83.0	5.9
4	73.1	2.1	61.4	4.4
2	51.0	1.5	40.0	2.8

Table 4
Comparison of dyeing and fastness properties of wool coloured with Nyloset N2R (C), dye A (1:1 2,5 DABSA: resorcinol) and dye B (1:10 2,5 DABSA: resorcinol). All dye mixtures were used at 8% v/v concentration in the dyeing bath.% STR-WSUM obtained with the enzymatic dyes is expressed as relative percentage respect to the commercial dye Nyloset N2R. Standard deviations among three replicates were less than 5%.

Dye	Dyeing properties		Colour Fastness			
	% STR-WSUM	K/S	Light	Washing	Rubbing	
					Wet	Dry
C	100	4.4	4/5	4/5	4/5	4/5
A	44.6	2.8	2/3	4/5	4/5	4/5
B	127.1	7.1	2/3	4/5	3	4

3.3.2. *Wool*

The best conditions for wool dyeing were analysed using different percentages of nanofiltered reaction products for each dyeing bath (Table 3). The CIELAB coordinates of the 8% dyed fabric were determined. The two synthesized dyes generated an almost similar tone, with a darker colour obtained in the reaction B. Both dyeing efficiency and colour strength increased with dye concentrations with 8% displaying the best performances. Fig. 4 shows that for wool dyeing, after the first bath, a discrete amount of both dyes A and B still remains in the dyeing solution, as already observed for nylon.

Properties of wool swatches coloured with 8% solutions of dyes from reactions A, B and Nyloset N2R were compared in Table 4. The dye A displayed the worst dyeing properties in terms of both dyeing efficiency and colour strength, whereas the dye B showed a better behaviour with respect to Nyloset N2R. Comparing fastness properties of wool swatches dyed with Nyloset N2R and both polymeric dyes A and B (Table 4), the whole fastness results are promising and in accordance with those obtained with nylon.

4. Discussion

The laccase catalysed coupling of aromatic compounds, such as phenols and diamines, to obtain coloured dimeric, oligomeric or polymeric compounds, has been already applied for textile dyeing [11,12,15,16]. In these reports, cotton and wool have been dyed using catechol coupled to 2,5-DABSA through an *in situ* process. A permanent colouration of cotton has been obtained through pigment fixation of the insoluble coloured product on the fibre [15]. Further improvement has been obtained introducing a pre-functionalization step of cotton, in which aromatic amine moieties of 2,5-DABSA have been attached to tosylated cotton and then cou-

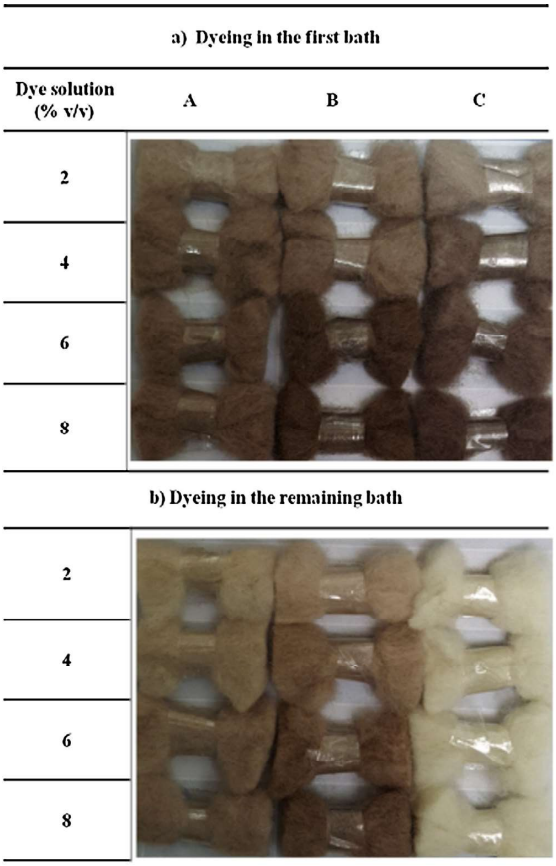

Fig. 4. Wool dyeing efficiency in the remaining bath. a) Wool dyed with different concentrations of dyes (2 ÷ 8% v/v) synthesized from reactions A (1:1 2,5 DABSA: resorcinol), B (1:10 2,5 DABSA: resorcinol) and the reference commercial dye Nyloset Brown N2R (C); b) wool dyed with the remaining bath derived from each dyeing processes reported in the panel a).

pled and copolymerised with catechol [12]. Effective wool dyeing has also been verified through the *in situ* reaction of 2,5-DABSA with catechol as well as with its isomer, resorcinol [11]. Different hues and depths of shades have been achieved by using the two isomers, reflecting the different laccase reactivity toward them.

In this context, our work aims at bridging the gap between the current research on enzymatically synthesized dyes and their concrete exploitation in an already existing industrial workflow. In fact, polymeric dyes were obtained through a laccase assisted reaction and after which applied to different fibres in the conditions routinely used for textile dyeing at Setaş facilities. The process was carried out without any dyeing auxiliaries. Two ratios of 2,5-DABSA and resorcinol were tested, and the products of both ratios at the highest substrate concentration (respectively dyes A and B) were scaled up for the colouration of different textile fibres. The two mixtures of dyes are effective in the dyeing of nylon and wool, while acetate, cotton, PES, and acrylic are faintly coloured. Results obtained on cotton are in accordance with previous findings indicating the need for a modification of fibre surface to achieve an improvement of dyeability [12]. To the best of our knowledge this is the first attempt to exploit such dyes on nylon fibre, whereas their application on wool has been already achieved through an *in situ* laccase based process [11]. It is noteworthy that fibre containing amino groups, such as wool and nylon (polyamide), display a higher uptake of enzymatically synthesized dyes. In particular, reported results indicate that dye B binds to wool with higher affinity respect to nylon. A similar behaviour is observed for the commercial dye Nyloset N2R. On the other hand, dye A displays almost the same affinity towards both fibres.

The superior colour strength of dye B towards both nylon and wool (Tables 2 and 4) indicates that a higher amount of this dye is bound to the fibres, although this observation seems to be in contrast with the results of the second dyeing bath (Figs. 3 and 4). This discrepancy is explainable considering that the enzymatically synthesized dyes may be a mixture of molecules with different dyeing properties competing in the interaction with the fibre. Thus, the presence of secondary products may interfere with the interaction of the dye in the first bath and/or contribute to the textile colouration in the second one.

5. Conclusions

In this paper the HRP POXA1b fungal laccase has been successfully exploited in a green process for dye synthesis thanks to its outstanding ability to work at alkaline pH and high temperature [21]. Aiming at addressing the industrial needs in terms of productivity, dye synthesis has been carried out at high substrate loading.

Two different bio dyes have been synthesized choosing two ratios between dye precursors, 1:1 (dye A) and 1:10 (dye B) 2,5 DABSA: resorcinol. Both dyes have proved to efficiently colour nylon and wool fibers, with dye B being a better candidate respect to dye A for textile colouration. In fact, increasing resorcinol concentration translates into the synthesis of a better performing dye both in terms of dyeing efficiency and colour strength. Both nylon and wool fibres coloured with the two bio dyes display good and comparable end quality and "performances during use". However, both dyes exhibit a week light fastness as a consequence of the already observed light initiated degradation [15].

Dyeing processes accomplished with the two bio dyes on both fibers exhibit a non exhaustive dye uptake after the first bath. This behaviour is explainable considering the presence of different colored products in the reaction mixtures which compete in the interaction with the fibers. Thus, the main obstacle to the use of the herein developed processes consists in the low purity and homogeneity of the produced dyes.

As future perspective, it remains to be elucidated if the cost of the purification of dye species could be balanced by an effective improvement of the dyeing performances. Moreover, this obstacle may be also circumvented by modifying the standard dyeing conditions applied in the textile industry (i.e. tuning time and temperature of incubation, as well as the ratio between g textile/mL dyeing bath) to promote the exhaustion of the dye in the first bath.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2016.11.016.

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5.2 Laccase-based synthesis of an innovative dye for protein gel staining

5.2.1 Introduction

Polyacrylamide gel electrophoresis (PAGE) is a reliable and widely used technique for the separation, identification, and characterization of proteins and protein mixtures. Broad application of electrophoresis techniques has required the development of detection methods that can be used to visualize the proteins separated on polyacrylamide gels. Two of the most routinely utilized methods are Coomassie Brilliant Blue R-250 (CBBR) staining and silver staining (Patton *et al.,*2002). Despite its higher sensitivity with respect to CBBR, silver staining is a time consuming procedure. As a fact, CBBR staining is a widely used method during common laboratory practices.
In this work, an enzyme based approach was tested for the synthesis of an α-naphthol based dye to be used as an alternative to CBBR for the protein gel staining To this aim the high redox potential POXA1b laccase was exploited for the α-naphthol / 2,5-dabsa coupling reaction. The resulting dye, named SIC_RED, was compared to the commercial CBBR in terms of sensitivity and ease of use. Moreover, the economic feasibility of the dye production was evaluated analyzing the cost of the synthesis process.

5.2.2 Materials and methods

5.2.2.1 Materials
Recombinant POXA1b laccase from *P. ostreatus* was expressed in the yeast host *Pichia pastoris*. All reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO) and Thermo Fisher Scientific Inc.

5.2.2.2 Laccase activity
Laccase activity was determined using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate at 420 nm (ε_{420}=36×103 M^{-1} cm^{-1}). The assay mixture contained 2 mM ABTS, 0.1 M Na-citrate buffer, pH3.0 (Childs *et al.,* 1975). Determination of laccase activity towards 2,5-dabsa and α-naphthol was performed by estimating increase in absorbance at around 340 and 800 nm after an incubation of 6 hours at 25°C. Assay mixture contains 1U/mL of POXA1b laccase (estimated using ABTS as substrate), and 10 mM of α-naphthol, 2 mM 2,5-dabsa or their mix in 50% v/v acetone.

5.2.2.3 Dye synthesis
Reaction conditions for dye synthesis were defined analyzing: four different ratios between the precursors; two concentration of POXA1b enzyme; two different reactions times. All the reactions were carried out in 50% v/v acetone at 25°C. Four ratios of α-naphthol: 2,5-dabsa were tested (1:1, 2:1, 5:1 10:1), using 2, 4 10 and 20 mM α-naphthol and the corresponding concentrations of 2,5-dabsa, with 1 and 2 U/mL of POXA1b analyzed after 6 and 24 hours of reaction. The absorbance spectra (340-800 nm) of the dye were registered at 25 °C using a Jasco V-530 UV/Vis spectrophotometer. Spectra recorded in the absence of enzyme were used as blank. Reaction products were loaded on a thin layer chromatography (TLC) after 6 and 24 hours. The mobile phase of TLC was 2:4:1:3 iso-propanol: ethanol: ethyl acetate: water, whereas the visualization was obtained through UV lamp and visible light.
SIC_RED was dried under nitrogen flux. The storage stability of the dried powder was determined storing the powder at room temperature and recordering the spectra of the daily re-suspended powder in 50% v/v acetone. The absorbance spectra (340-800 nm) of the dye was registered during a period of 60 days at 25 °C. The storage stability of the dye in the working solution (50%methanol-10% acetic acid) was calculated estimating the variation of the spectra after a period of 45 days at 25°C.

5.2.2.4 ATR-FTIR analysis
The dye suspension was transferred to a glass column (1.5 × 30 cm) packed with 40 g of silica gel 60, particle size 0.06–0.2 mm, and eluted with iso-propanol: ethanol: ethyl acetate: water (2:4:1:3). Different fractions were collected and the most colored ones evaporated to dryness. The samples were analyzed by FTIR as follows: attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of powder samples were acquired using a Nicolet 5700 spectrometer provided with a MCT detector and a GladiATR single-bounce monolithic diamond ATR accessory. The spectra were recorded in the 4000–400 cm^{-1} wavenumber range, at 4 cm^{-1}resolution, by averaging 124 scans.

5.2.2.5 Protein staining of a SDS-PAGE gel
Electrophoresis was carried out on polyacrylamide slab gels (60 x 80 x 0.75 mm) containing 0.1% SDS using the discontinuous buffer system of Laemmli (Laemmli, 1970). The proteins used for the analysis were: lysozyme, bovine serum albumin (BSA), ovalbumine and Vmh2 hydrophobin. Protein concentrations were determined by Bradford's method using Bio-Rad protein assay kit with BSA as a standard (Bradford, 1976). In the case of the Vmh2 hydrophobin the concentration was evaluated using the Pierce 660 nm protein assay kit (Thermo Scientific, Waltham, MA) using BSA as the standard protein. Protein samples were dissolved into buffer containing 62 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 5% mercaptoethanol, 0.005% bromophenol blue. The samples were heated at 100 for 5 min and then cooled to room temperature before electrophoresis. The running buffer was 0.025 M

Tris-HCl, 0.2 M glycine, 0.1% SDS, pH 8.3. Stacking gels (4.5%) of 1.5 cm in length was overlaid on separating gel of 12.5% polyacrylamide with an acrylamide: bis-acrylamide ratio of 29: 1. Gels were run in a Mini-Protein II dual slab cell (Bio-Rad Lab., Hercules, CA, SA). Electrophoresis was performed at constant current of 15 mA per slab gel.
Silver staining was performed as reported in literature (Chavallet *et al.,* 2006).
CBBR staining was performed in 20 ml of 0.1% CBBR in the working solution (50% methanol 10% acetic acid) for 60 min. The gels were de-stained by two consecutive washing steps with 50 ml of washing solution (5% methanol, 7.5% acetic acid), for 2 hours.
SIC_RED staining was performed in 20 ml of 0.1%, 0.05%, 0.02% and 0.01% SIC_RED in the working solution (50% methanol ÷ 10% acetic acid) for 100 min. The gels stained with 0.1% and 0.05% of SIC_RED were de-stained in the washing (5% methanol, 7.5% acetic acid) for 5 minutes, whereas the gels stained with 0.02% ans 0.01% SIC_RED were not de-stained.

5.2.2.6 Densitometric analysis
Quantification of the protein band intensity was performed by ChemiDoc™ MP Imaging System (BioRad) integrated with Image Lab Software. The values acquired were reported as pixel intensity inside the band volume and converted in percentages respect to a reference sample of CBBR.

5.2.2.7 Cost estimation methodology at production scale
Cost analysis of dye production was performed by implementing the process (fermentation, biomass separation and enzyme concentration) in Superpro Designer 9.0 (Intelligen Inc.). Mass and energy balances have been performed to evaluate electricity consumption and utilities duties at each unit operation.
The chosen scenario describes the case of BIOPOX s.r.l.: a small-medium enterprise, hosted by the Department of Chemical Sciences of the Università degli Studi di Napoli Federico II (Italy) and sharing big equipments with it. According to that, Italy was chosen as location for fixing the cost of labor, electricity and other utilities as reported in Table S1.
Capital (CAPEX) and Operating Expenditures (OPEX) were calculated according to the procedure reported in Table S2. Capital investment for infrastructures (electrical, building land improvement and service facilities) are not considered, according to the BIOPOX scenario. Costs of chemicals were taken either from bulk quotation gently provided by Sigma-Aldrich or VWR and reported in Table S3. A batch schedule has been implemented for operations associated to each unit, accordingly to the procedures reported in previous sections. Major equipment is depreciated assuming 10 years lifetime of the process with an 8% interest rate. According to that, the depreciation quote of each equipments was partially charged on the CAPEX of BIOPOX in dependence of the usage time. A 330 days production per year was adopted to take into account maintenance.
Process scale was initially fixed to a working fermenter volume of 1 L and then future scenarios were modeled scaling it up to 100 L. Cost of equipments were scaled adopting the 6/10 exponent rule (Heinzle *et al.,* 2006). Wastewater treatment was fixed to 0.4 € m^{-3} (Heinzle *et al.,* 2006).

5.2.3 Results and discussions

5.2.3.1 Design of the dye synthesis

Laccase is known for its ability to catalyze the oxidation of phenolic and amine groups (Giardina *et al.,* 2009). These molecules can react with laccase via one-electron oxidation, followed by further enzymatic or non-enzymatic reactions of the newly formed radicals.

The activity of the recombinant laccase POXA1b was first assayed towards 2,5-dabsa and α-naphthol precursors in 50% acetone. The reactions were carried out in acetone to sustain both substrates and product solubilization (Aktas *et al.,* 2000).

POXA1b was able to oxidize either the 2,5-dabsa and the α-naphthol (Fig. 1). In fact, a remarkable increase of absorption intensity was measured, with peaks centred at around 340 and 460 nm for α-naphthol and 2,5-dabsa.

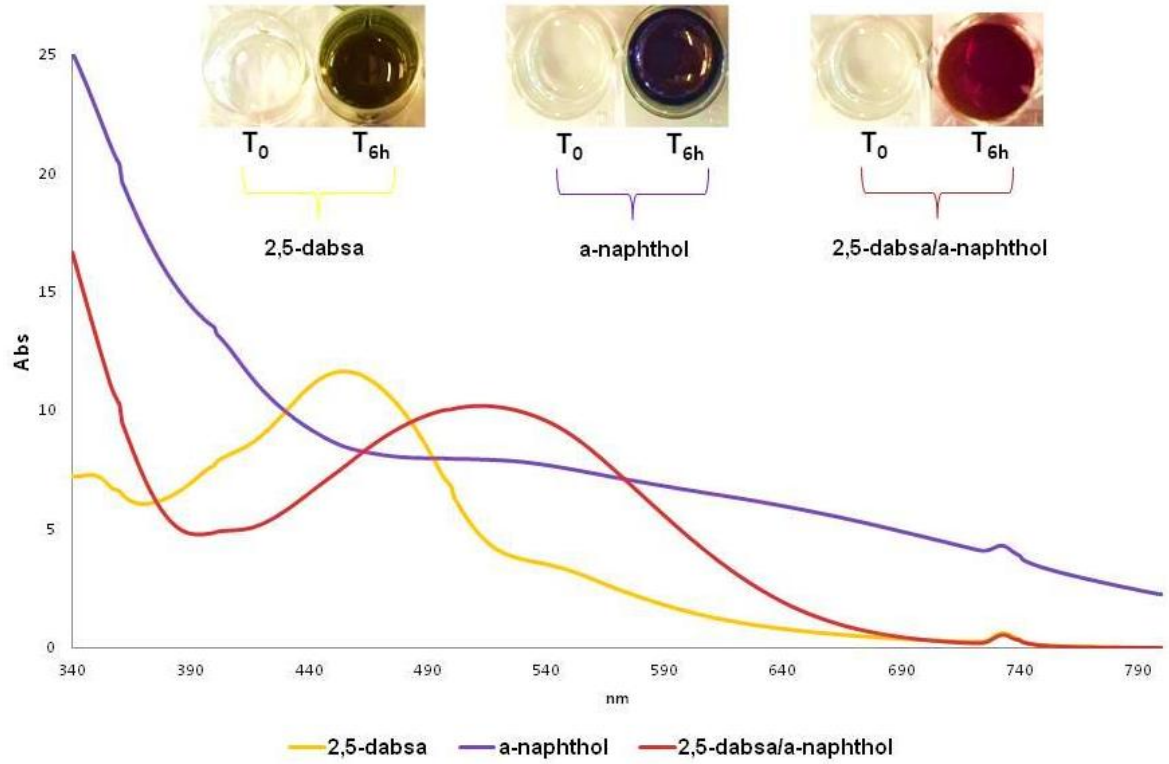

Fig. 1. Precursors spectra analysis. Absorption spectra (340–800 nm) recorded after 6 h reaction with 1UmL^{-1} of POXA1b laccase with 2,5-dabsa, α-naphthol and their mix. Standard deviations among three replicates were less than 5%.

The formation of a new coloured product, SIC_RED, was proven by the appearance of an absorbance peak at around 500 nm.

The possible reaction mechanism of the two precursors involves three main steps (Fig. 2): *(i)* enzymatic oxidation of α-naphthol to produce highly reactive radicals able to spontaneously polymerize to poly-naphthol (Islam *et al.,* 2014; Xu *et al.,* 2005); *(ii)* activation of 2,5-dabsa (promoted by enzymatic oxidation); and *(iii)* non-enzymatic cross coupling between the activated donor and the poly-naphthol to generate the hetero-polymer (Vicente *et al.,* 2016).

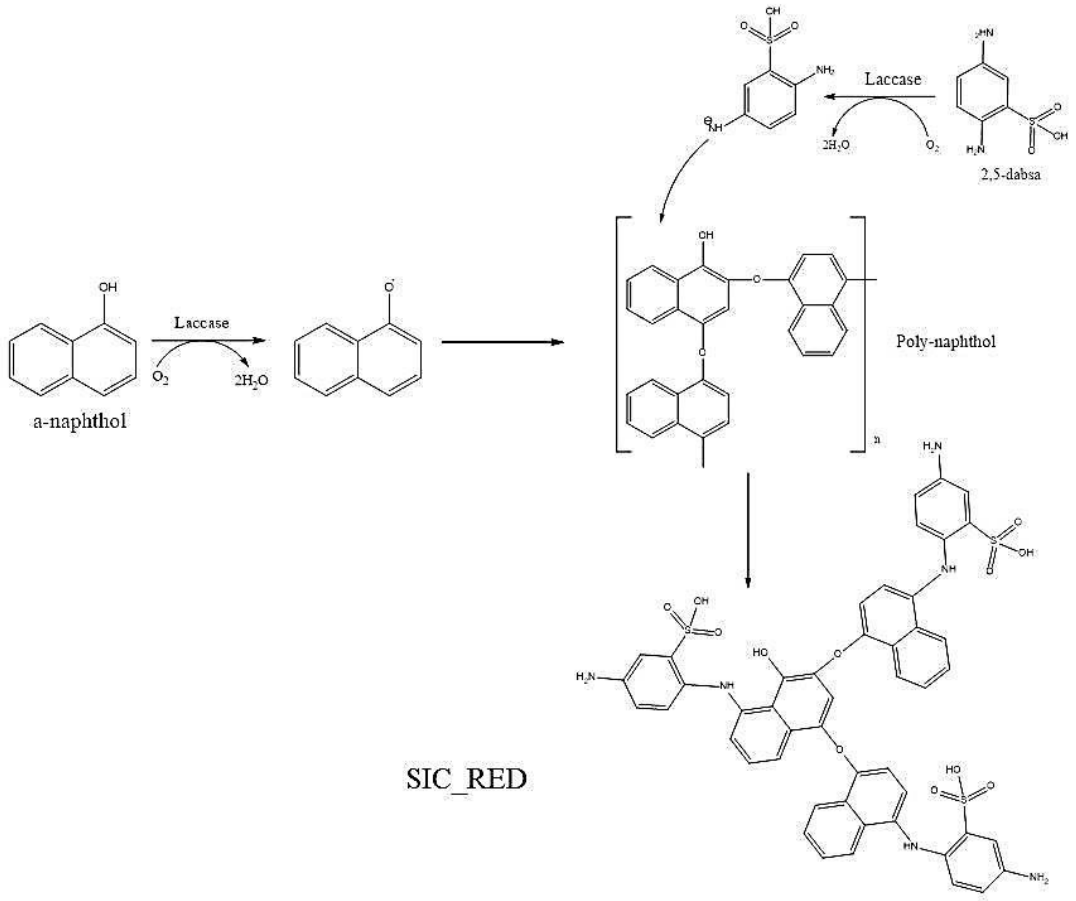

Fig 2 Suggested reaction route for SIC_RED dye synthesis by laccase. (adapted from Ref. Calafell *et al.*, 2007 and Xu *et al*., 2005).

Reaction conditions for dye synthesis were set-up evaluating the effect of the ratio between the two precursors (α-naphthol: 2,5-dabsa 1:1, 2:1, 5:1 10:1), the concentration of POXA1b enzyme (1 or 2 U/mL), and the reaction time (6 and 24 hours).

Thin Layer Chromatography (TLC) was used to follow dye synthesis. The criteria utilized for the selection of the best reaction conditions were: *(i)* consumption of both precursors and *(ii)* homogeneity of the products.

The results obtained by TLC (Fig.3) suggested that the condition 1U/mL in the ratio 5:1 at 6 hours showed an visible higher homogeneity of the dye product and the complete consumption of 2,5-dabsa, whereas in the case of α-naphthol a semi-completed consumption was observed. The same results were evident also in the condition of 2 U/mL of the enzyme and after 24 hours of reaction with both enzyme concentration, but in order to reduce the process cost the condition with a ratio of 5:1 α-naphthol-2,5-dabsa, 1U/mL of POXA1b and 6 hours of reaction was chosen for further characterization.

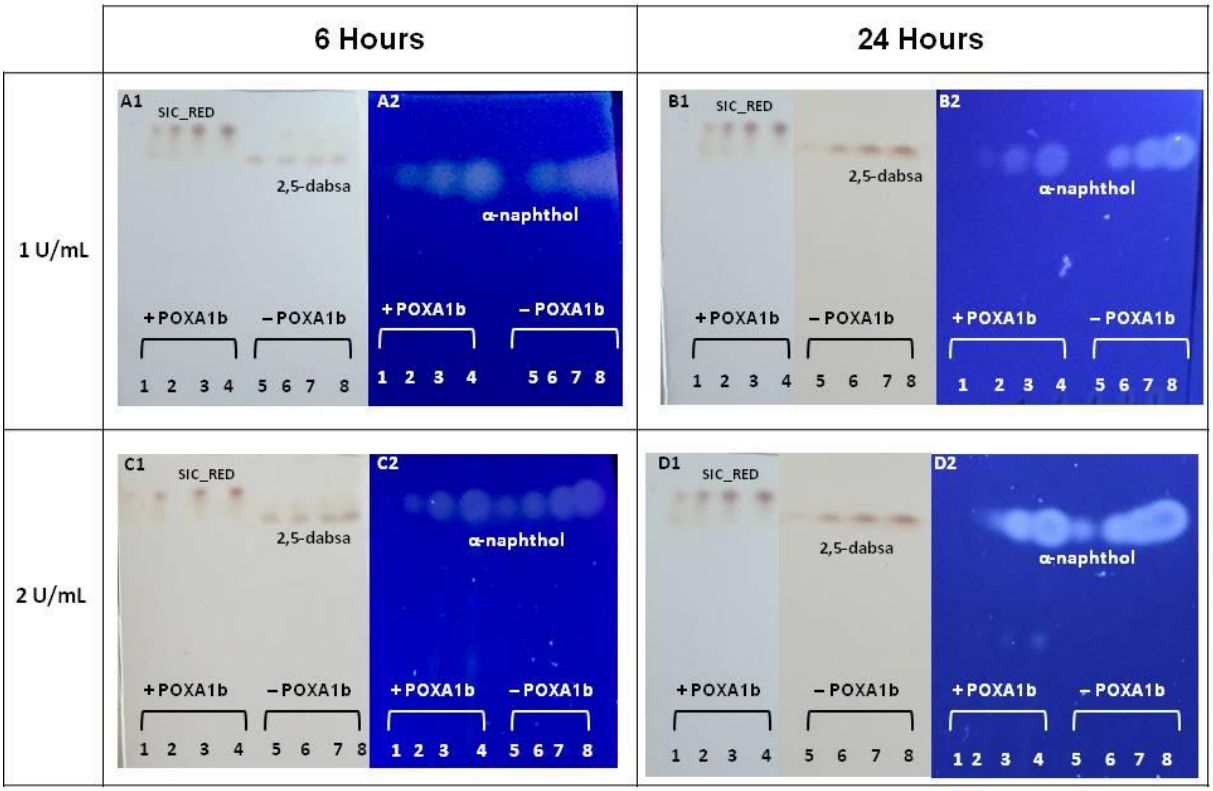

Fig. 3 TLC of α-naphthol/2,5-dabsa with POXA1b. 1) ratio 1:1; 2) ratio 2:1; 3) ratio 5:1; 4) ratio 10:1. TLC of α-naphthol/2,5-dabsa without laccase. 5) ratio 1:1; 6) ratio 2:1; 7) ratio 5:1; 8) ratio 10:1. A1, B1, C1, D1 TLC visualization by visible light. A2, B2, C2, D2 TLC visualization by UV light.

Spectrum of SIC_RED synthesized in the selected conditions is shown in Fig 4.

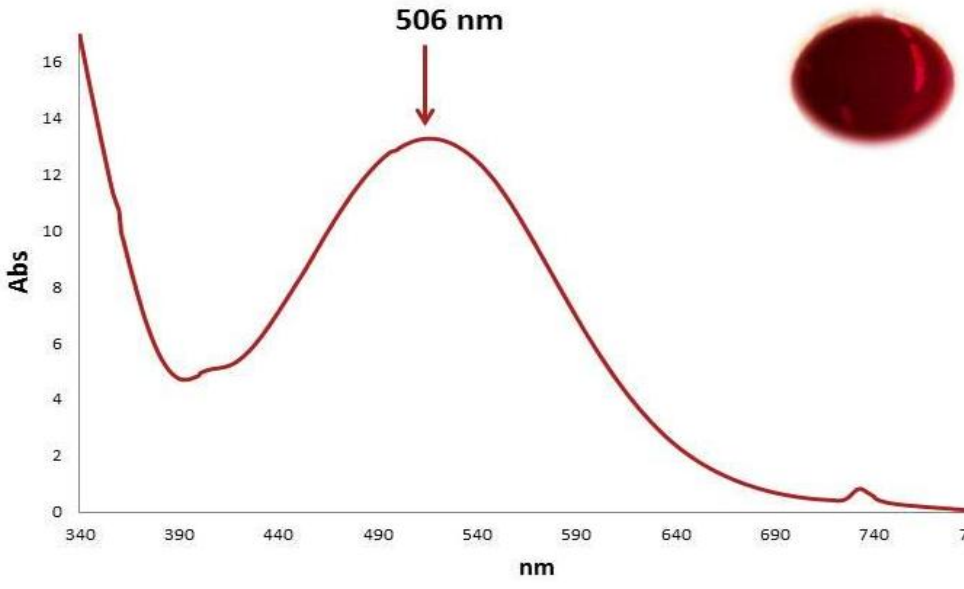

Fig. 4 SIC_RED dye spectra analysis. Absorption spectra (380–800 nm) recorded after 6 h reaction with 1 U mL^{-1} of POXA1b laccase.

SIC_RED was dried, purified on silica column and analysed by an ATR-FTIR (Fig. 5).

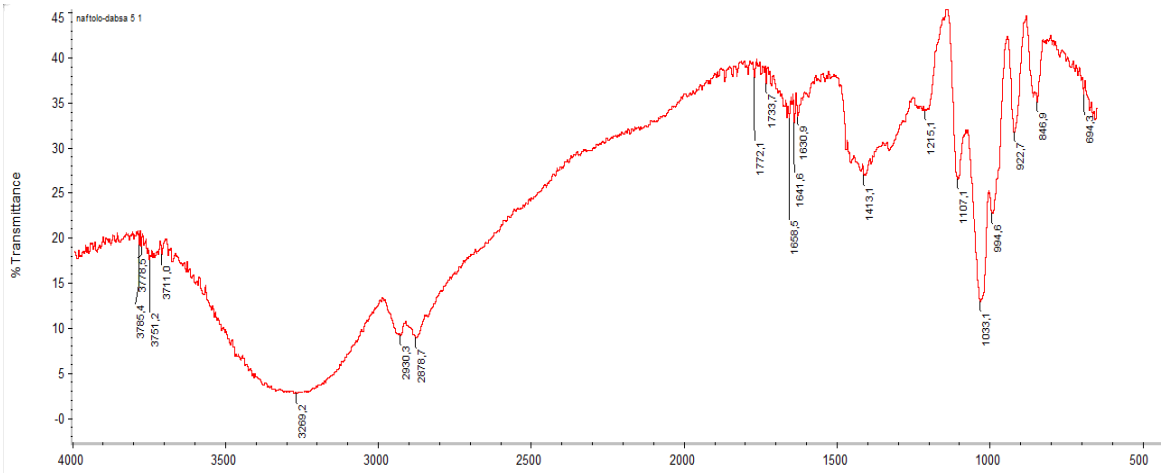

Fig 5 ATR-FTIR spectrum of the synthesized SIC_RED.

The overall absorbance of the dye in the region between 1400 and 1600 cm^{-1}, suggested a significant conjugation of aromatic rings through C-C, C-O-C and -NH- bonds (David *et al.,* 1996; Dubey *et al.,* 1998; Sanchez-Cortes *et al,.* 2001; Kramer *et al.* 2001; Aktas *et al.* 2003; Shaw *et al.* 2004). Further analysis of the dye product showed the following key peaks at 3300 and 1413 cm^{-1}. The broad band at 3300 is due to O-H phenolic stretching. The peak at 1413 cm^{-1} is possibly due to N-H bonding. Peaks at 1107 and 847 cm^{-1} could be assigned to S–O and S=O bond, respectively, due to the presence of 2,5-dabsa; peaks at 1033 cm^{-1} could be assigned to C-N bond indicating the occurrence of a C-N hetero-coupling (Hadzhiyska *et al.,* 2006, Calafell *et al.,* 2007, Sun *et al.,* 2013).

5.2.3.2 Exploitation of SIC_RED in protein gel staining

The potential similarity of the hypothesized structure of the SIC_RED with the CBBR (Fig 6) suggested to test the new dye for protein gel staining. As a fact, the presence of sulfonic acid groups (negatively charged at pH ~3), and of the hydrophobic poly-naphtol core confers to the molecule the ability to bind both basic and aromatic amino acids.

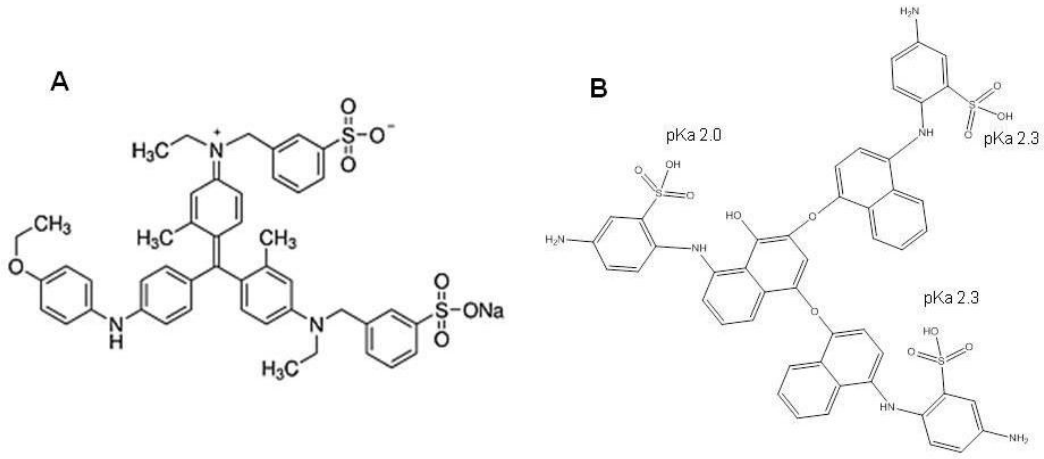

Fig. 6. Structure comparison between (A) Coomassie Brillant Blue R-250 (B) SIC_RED (The SIC_RED pKa was estimated through Epoch software (https://epoch.uky.edu/ace/public/pKa.jsp).

Before testing SIC_RED ability in protein gel staining, its solubility and storage stability were assessed. Dryed SIC_RED was resuspended in 50 % acetone and in the working solution for protein gel staining (Fig. 7). It is worth to note that the powder is efficiently solubilised in both conditions, although there is a dependence of the absorption spectra from the solvent used.

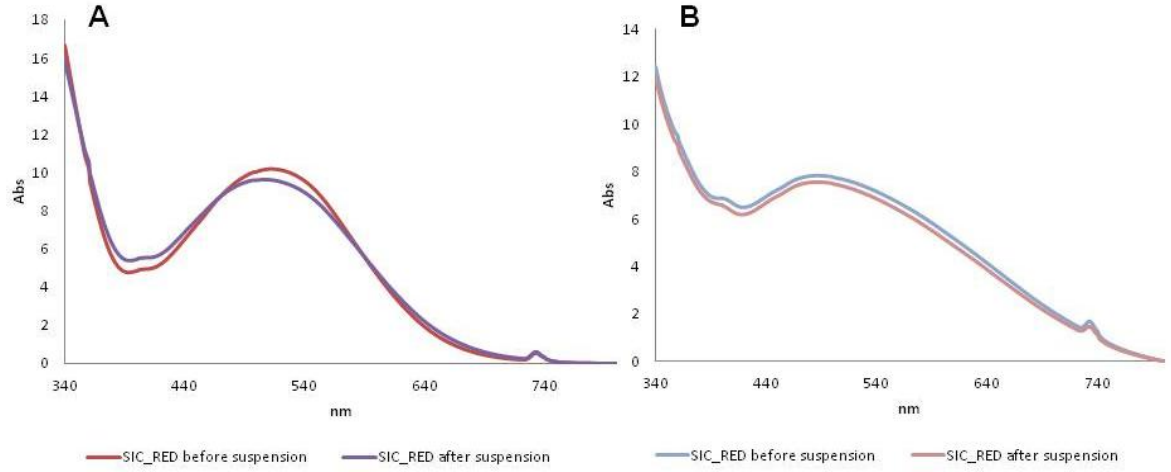

Fig. 7 Sic_REd solubility (A) resuspension in 50% acetone (B) resuspension in working solution

The storage stability of the synthesized product in the two different conditions was also evaluated: in the form of powder and after its resuspension in the working solution. The data indicated that the $t_{1/2}$ of the powder resuspend in the acetone was more than 2 months, while in the case of its resuspension in the working solution the $t_{1/2}$ was more than 45 days, rendering the product viable for exploitation in the target process.
SIC_RED performances in protein dyeing were compared with those of the commercial CBBR in terms of sensitivity and ease of use. Sensitivity of SIC_RED was also compared to that of silver staining procedure. Four proteins endowed with different percentage of positive charged residues (Tab. 1) were tested.

Tab.1 Percentage of positive charged residues in the selected protein.

Proteins	Arg (R) %	His (H) %	Lys (K) %	Percentage of positive charged residues (%)
Bovin Serum albumin (BSA)	4.3	2.8	9.9	17
Ovalbumin	3.9	1.8	5.2	10.9
Lysozyme	8.2	0.7	4.1	13
Vmh2	0.0	0.0	1.1	1.1

CBBR and SIC_RED exhibited a comparable tendency to bind protein with similar amino acidic compositions. As a fact neither of the two dyes was able to stain Vmh2 hydrophobin (Fig 8).

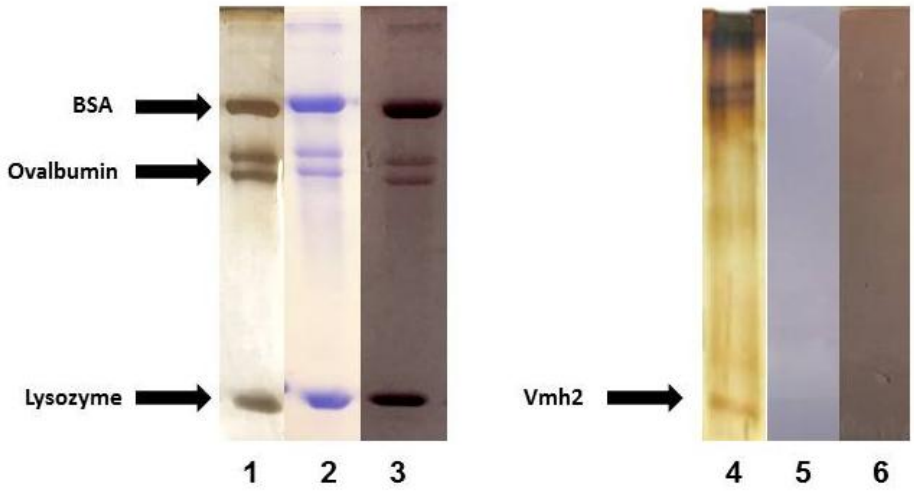

Fig. 8 Comparison of different protein staining methods on SDS-PAGE gel of BSA, ovalbumin, lysozyme and Vmh2 proteins: 1-4) Silver staining; 2-5) CBBR staining; 3-6) SIC_RED staining.

In order to define the optimal concentration of SIC_RED to be used in the working solution, four different conditions (0.01 ÷ 0.1 %) were compared respect to the CBBR commercial working solution (0.1 %, as suggested by the manufacturer) (Fig. 9).

	BSA protein quantities (µg)	
Dye concentration (%)	1	0.5
Coomasie 0.1	100%	100%
SIC_RED 0.1	106%	98%
SIC_RED 0.05	92%	88%
SIC_RED 0.02	114%	97%
SIC_RED 0.01	32%	31%

Fig. 9 Comparison of staining sensitivity between the CBBR standard concentration and different SIC_RED concentrations. Quantitation of the protein band intensity was performed by ChemiDocTM MP Imaging System (BioRad). Standard deviations among three replicates were less than 5%.

The data indicated that performances of SIC_RED were comparable up to a dilution of 0.02 %.

Sensitivity of SIC_RED staining of BSA used as standard at three different working solutions was assessed in comparison with those of CBBR (Fig. 10).

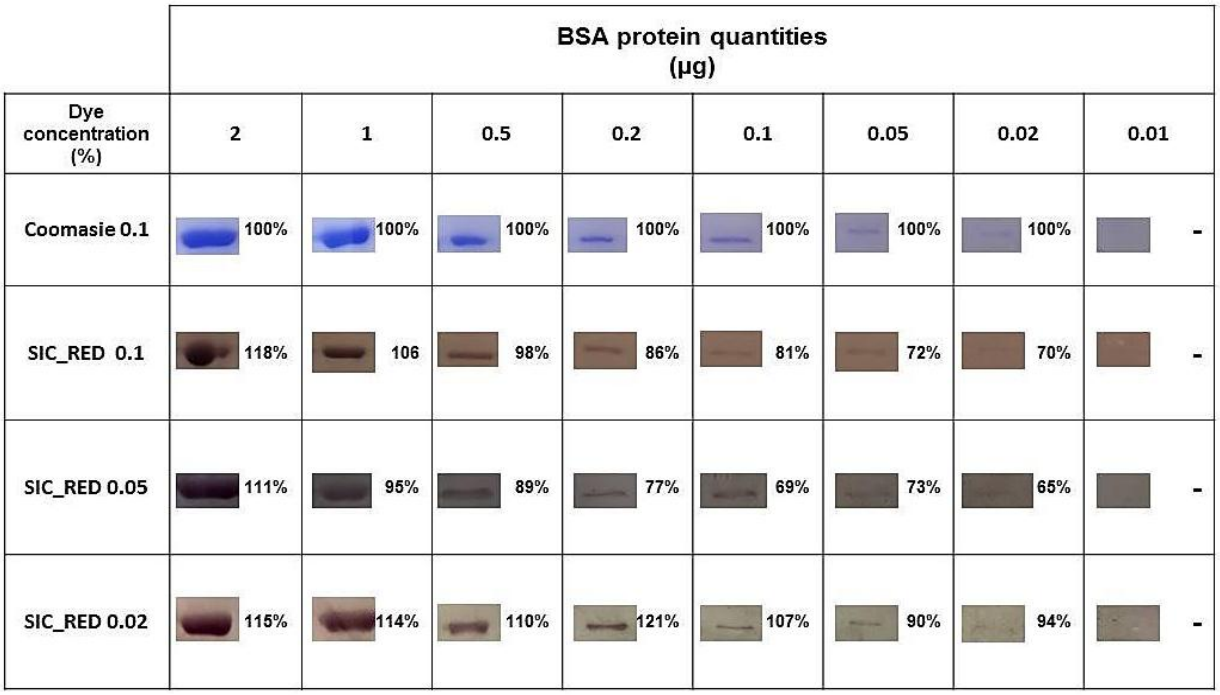

Fig. 10 Comparison of staining sensitivity between the CBBR and SIC_RED. Quantitation of the protein band intensity was performed by ChemiDocTM MP Imaging System (BioRad). Standard deviations among three replicates were less than 5%.

The data analysis indicated that the optimal SIC_RED concentration was 0.02%, allowing to reduce the dye concentration of up to five folds respect to CBBR, while maintaining the same protein sensitivity (~20 ng). This condition also speeds up the total procedure with a reduction of ~ 2 hours. In fact the corresponding gel background was completely clear avoiding the de-staining step.
In order to further improve SIC_RED staining process respect to the commercial CBBR, methanol concentration was reduced in the staining solution. It is worth to note that methanol helps the dye solubilisation simultaneously preventing the SDS-gel swelling. SIC_RED dye (0.02%) was dissolved in working solutions with reduced methanol concentrations (Fig. 11)

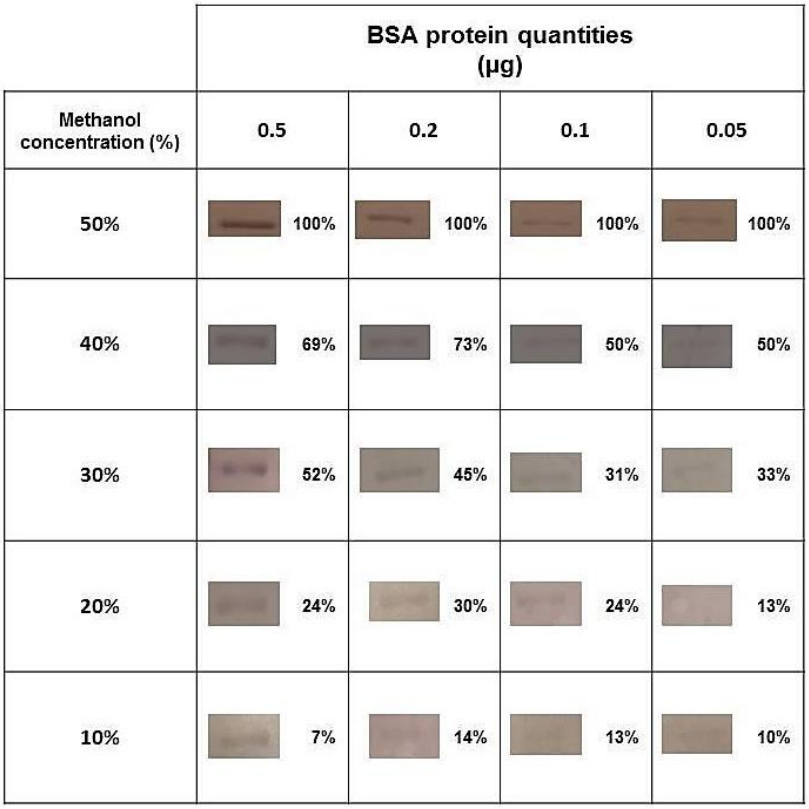

Fig. 11 Comparison of SIC_RED staining efficiency at different methanol concentrations. Quantitation of the protein band intensity was performed by ChemiDocTM MP Imaging System (BioRad). Standard deviations among three replicates were less than 5%.

A reduction of methanol concentration resulted in the reduction of the dye efficiency preventing its penetration inside the gel matrix and the consequent interaction with the protein.

5.2.3.6 Estimation of cost production

Figure 12 shows the cost breakdown for the dye at different volume scales and laccase unit costs. On a yearly basis, about 1200 batches can be performed, achieving a production rate of dye ranging from 1.2 up to 1200 kg. At a laccase unit cost of 1 € kU^{-1}, the unit cost of dye decreases from 33 to 2 € g^{-1}. It can be observed that a 1 L volume scale has the labor as bottleneck, but when increasing it 100x it becomes clear that the cost of the chemicals is predominant, counting for 81% of the unit production cost. The effect of the enzyme cost can be appreciated when decreasing it from 1 to 0.3 € kU^{-1}: the production cost of the dye is halved.

It has to be stressed that at these final operating conditions the cost of acetone mainly accounts for the chemical part. As future recommendations two improvements can be verified and eventually performed in the process: 1) the implementation of an ultrafiltration operation after the reactor in order to recover and recycle the enzyme to the reactor; 2) the implementation of a condensation on the off-gas from drying in order to recycle the vaporised acetone to the reactor.

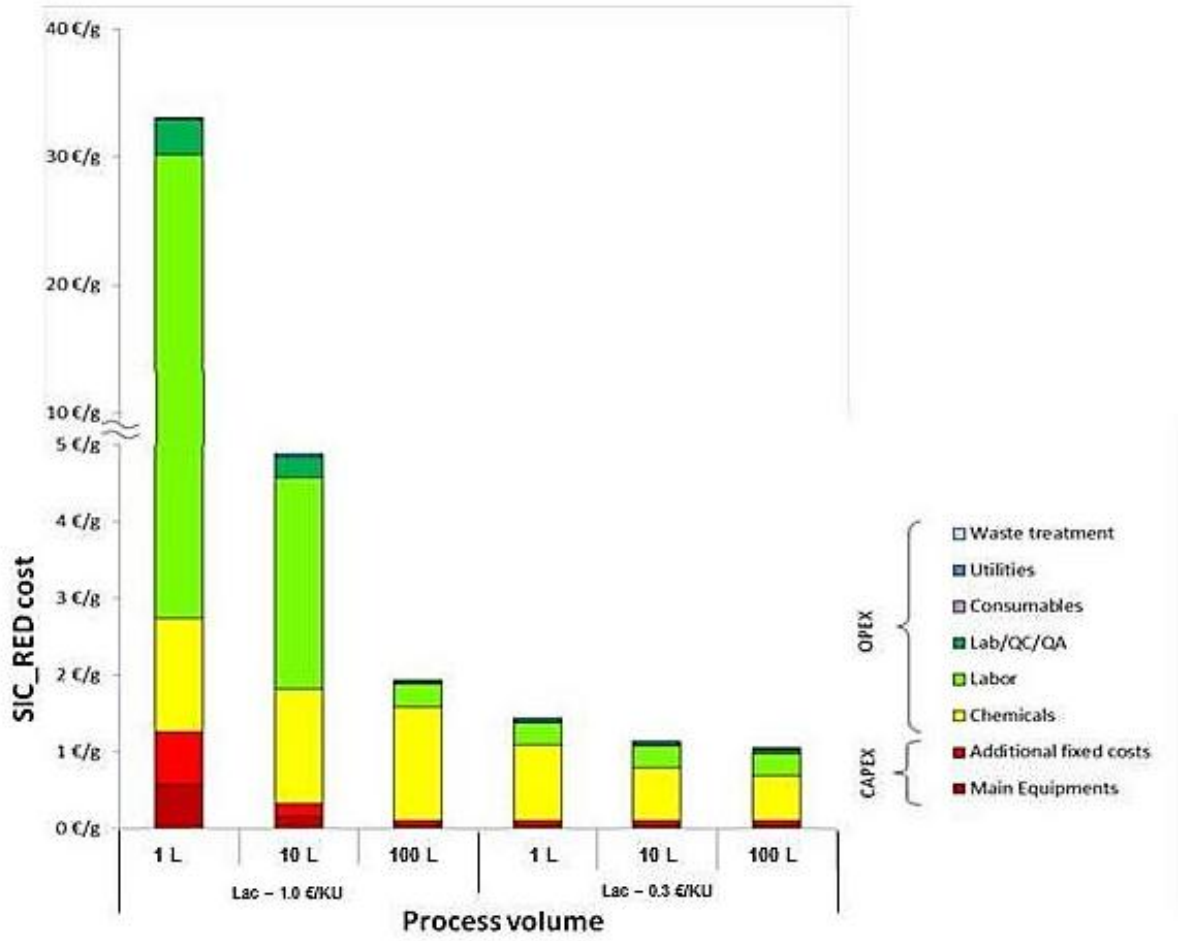

Figure 12 Cost breakdown for the dye production at different volume scales and unit cost of the enzyme

This result gives a clear indication that the process is competitive at a representative production scale of a small company, considering that the commercial cost of CBBR is generally about 2.4 € g^{-1}.

5.2.4 Conclusions

In this chapter the POXA1b fungal laccase has been successfully exploited in a "green" process for dye synthesis and applied in protein staining on polyacrylamide gel. Aiming at addressing the industrial needs in terms of productivity and eco-sustainability, dye synthesis optimization has been carried out. The synthesized SIC_RED was used for the protein gel staining, comparing its performances in terms of specificity and sensitivity with the commercial CBBR. This comparison showed that SIC_RED procedure allowed to reduce the dye concentration of up to five folds respect to CBBR, while maintaining the same protein sensitivity (~20 ng). This condition also speeds up the total procedure with a reduction of ~ 2 hours. Furthermore a cost analysis of the process was performed. The SIC_RED cost obtained (2 € g^{-1} ÷ 1.0 € g^{-1}), was comparable to the commercial one, making the process available for a possible commercialization.

5.2.5 References

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5.2.6 Supplementary materials

Table S 1. Unit cost of electricity, labor and utilities referred to the Italian scenario of industrial production

	Unit cost
Operator labor	28.8 € hr^{-1} *
Electricity	0.16 € kWh^{-1} **
Freon	0.18 € MT^{-1} ***
Chilled water	0.49 € MT^{-1} ***
Hot water	0.062 € MT^{-1} ***
Steam	14.8 € MT^{-1} ***

*source: Bureau of Labor Statistic - www.bls.gov. Lumped cost considering employer contribution, supervision and

**source: Eurostat - http://ec.europa.eu/eurostat/statistics-explained/index.php/Energy_price_statistics

***source: Data are taken from SuperPro databank and linearly scaled by ratio of electricity cost in US and in Italy

Table S 2 – Procedure for estimating CAPEX and OPEX

			Cultivation
TOTAL CAPITAL INVESTMENT	Direct Cost (DC)	Major equipment cost	MEC
		Installation costs	30% MEC
		Instrumentation and control	40% MEC
		Piping	20% MEC
		Insulation	3% MEC
		Electrical	0% MEC
		Buildings	0% MEC
		Land improvements	0% MEC
		Service facilities	0% MEC
	Indirect Cost (IC)	Construction expenses	10% DC
		Engineering and supervision	10% DC
	Other Cost (OC)	Contractor's fee	5% (DC+IC)
		Contingency (Major equipment)	5% (DC+IC)
	Direct fixed capital cost (DFC)		DC+IC+OC
	Working capital		OPEX first month of operation
	Start-up and validation		5% DFC
	Total capital investment		DFC + working capital + Start-up and validation
CAPEX	Depreciation		(DC+IC+OC)/15 years
	Interest		8% of depreciation
	Property tax		2% of depreciation+interest
	Insurance		1% of depreciation+interest
	Purchase tax		5% of depreciation+interest
OPEX	Energy		Calculated from MEC consumption
	Labour		Salaries + Employer's contribution +Supervision
	Raw materials		Calculated from mass balances
	Utilities		Calculated from MEC consumption and mass balances
	Laboratory/Quality control/Quality assurance		10% Labour cost
	Wastewater treatment		Calculated from mass balances
	Consumables		Calculated from MEC design
	Others	Maintenance	5% DFC
		Operating supplies	0.4% (Electricity + Raw materials + Utilities)
		Contingencies	15% (Raw materials + Utilities)
		Overheads	55% (Labour + Maintenance)

Table S 3 – Unit cost of chemicals

Chemical	Unit cost
α-naphthol*	30 €/Kg
2,5-dabsa*	90 €/Kg
Acetone**	6 €/L
POXA1b laccase	1-0.3 €/KU

*Sigma-aldrich
**VWR

CHAPTER 6

Laccase-mediated cotton functionalization

To be submitted to Biofouling

Laccase-mediated cotton functionalization

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Abstract

In this study a laccase-based process for the functionalization of cotton textile was investigated. The process has been designed starting from resorcinol and 2,5 diaminobenzensulfonic acid as precursors. Due to its peculiar stability at alkaline pH, POXA1b laccase from *Pleurotus ostreatus* was used to catalyze the synthesis and simultaneously binding of a phenol-based coating onto cotton fiber. Laccase promoted grafting was found effective in cotton colorization as well as in conferring interesting properties to the fiber. The anti-adhesive effect against *Gram positive* and *Gram negative* bacteria was verified The functionalized cotton fibers also showed a significant antioxidant activity. An innovative multifunctional textile was achieved, with potential applicability in home furnishings, clothing and medical textile.

Keywords: laccase, cotton, anti-oxidant, anti-adhesive

6.1 Introduction

World fiber consumption has increased over several decades. From 1950 to 2008, the *per capita* consumption increased from 3.7 kg to 10.4 kg, and through continuous development, it recorded in 2014 a demand of 55.2 million tons (122 billion pounds) of synthetic fibers, in addition to the natural fibers, including cotton and wool, which have a demand of 25.4 million tons (FAO and ICAC 2011). In particular, natural fibers like cotton play an important role in several industries throughout the world, being used every day for different purposes such as in domestic home furnishings, automotive textiles, air filters, thermal and mechanical protection, sport equipment, healthcare and hygienic applications (Collier *et al.,* 2001, Singleton 2013, Shahidi *et al.,* 2012). Cotton is the world's most widely used natural fiber being versatile in its application and easily available. However cotton dyeing is still a concern for the textile industry due to its low affinity for the most applied class of dyes, the anionic ones. In fact, the coloration of cotton involving anionic dyes requires vast amounts of salt for efficient dye utilization and fastness requirements. As a result, large amount of salt is discharged in the dye bath effluent (Tsegai, African journal). Carefully selection of dyes and chemicals, optimized procedure and equipment design as well as the reuse of exhaust dye-bath can reduce the pollution impact of textile wet processing industries (Bashar 2013). However, these approaches require chemicals and auxiliaries, as well as the setting up of new procedures and equipment, thus adding costs to the dyeing process. Modifying the cotton fiber to increase its dye uptake is one of the route to overcome the drawback associated to cotton dyeing.

Due to their large surface area and ability to retain moisture, cotton also acts as carriers for microorganisms, such as pathogenic and odor generating bacteria, mould and fungi, causing damage to clothing, strength loss, staining, discoloration and also skin diseases. As a fact, under certain conditions the presence of carbohydrates in the cotton fiber operates as nutrients and energy source to microbes (Gao and Cranston 2008; Schindler and Hauser 2004; Srivastava *et al.,* 2011). Antimicrobial finishes maintain hygiene and enable to avoid infection from pathogens especially in hospitals, nursing homes, schools, hotels, and crowded public areas. Most of the antimicrobial agents used in commercial cotton are biocides acting in different ways to control of the microbial growth: from preventing cell reproduction, blocking of enzymes, reaction with the cell membrane to the destruction of the cell walls and poisoning the cell from within (Schindler and Hauser 2004). Another method used to reduce the microorganism growth is preventing the cell adhesion to the material surfaces.

The latter includes both modification of surface chemistry by the linkage of molecules displaying anti-adhesion properties (PEG, zwitterions), as well as the use of enzymes or molecules able to degrade the biofilm matrix or interfere with its formation (Aswathanarayan and Vittal 2013, He *et al.,* 2012 and Ivanova *et al*., 2015). The array of antibacterial surfaces reported in scientific literature can be also categorized according to the modifications they have been subjected, such as physical modification, addressing surface topography, or chemical modification, including surface derivatiation and polymerization (Hasan *et al.,* 2013). Most of these strategies are costly to be scaled-up and work well on particular materials' surfaces compatible with the specific conjugation, but lack efficacy on broad ranges of materials.

In this complex picture, there is a quest for derivatisation technologies which are versatile, cost effective and easily scalable to industrial scale

An environment-friendly approach that is becoming increasingly used in textiles in the last years is the promotion of specific reactions catalyzed by enzymes. Laccases are known for their ability to modify various surfaces through grafting of phenolic moieties, conferring properties such as antimicrobial, hydrophobicity and antioxidant (Piscitelli *et al.,* 2011).
In this work an *in situ* dyeing process was tested for cotton coloration using the enzyme POXA1b and 2,5-dabsa and resorcinol as precursors. An innovative multifunctional textile was achieved, with potential applicability in home furnishings, clothing and medical textile.

6.2 Material and methods

6.2.1 Materials
Recombinant POXA1b laccase from *P. ostreatus* was expressed in the yeast host *Pichia pastoris*. All reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The textile fabrics were furnished by Setas colour centre (Turkey) in the frame of INDOX European Project (KBBE-2013-7-613549).

6.2.2 Laccase activity
Laccase activity was determined using 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate at 420 nm ($\varepsilon 420=36\times10^3$ M^{-1} cm^{-1}) as previously reported (Childs *et al.,* 1975).

6.2.3 Cotton functionalization through laccase based process
Cotton swatches (1cm x 1cm) were submerged in buffer solution of 100 mM Tris-HCl pH 8 containing 25 mM 2,5-dabsa, 250 mM resorcinol or their mix with 1 U/mL of POXA1b. Each cotton swatch was incubated in 10 mL of reaction mixture. The solutions were incubated for 9 hours at 60°C under agitation (220 rpm) (Pezzella *et al.,* 2016). A cotton swatch was incubated for 9 hours at 60°C under agitation (220 rpm) in 100 mM Tris-HCl pH 8 as control. At the end of the incubation the cotton swatches were washed twice in order to remove the unbound molecules. The samples were dried at 60°C for 15 hours.
To simulate washing conditions, the samples were placed in a sterile solution of water and 0.35% v/v of commercial laundry detergent (General, Henkel). The samples were vortexed for 10 minutes at 30°C. At the end of the washing step, the swatches were cleaned twice in sterile water for 10 minutes and dried at 60°C for 15 hours.

6.2.4 ATR-FTIR analysis
The samples were analyzed by FTIR as follows: attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra of cotton samples were acquired using a Nicolet 5700 spectrometer provided with a MCT detector and a GladiATR single-bounce monolithic diamond ATR accessory. The spectra were recorded in the 4000–400 cm^{-1} wavenumber range, at 4 cm^{-1} resolution, by averaging 124 scans.

6.2.5 Anti-adhesion test
The anti-adhesion test is a variant of ‘‘AATCC Test Method 100-2012, Antibacterial Finishes on Textile Materials: Assessment of'". The bacteria cells used for the test were Gram negative *Escherichia coli* strain Top 10 and Gram positive *Bacillus subtilis*. Samples swatches were dried and placed into a sterile container. Three swatches were used for each assay condition. A cell suspension was prepared by diluting a microbial growth in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4). These two suspensions were placed onto the top of the swatches (1cm x 1cm) at a concentration of 10^5 CFU/mL. The inoculated swatches were incubated for 24 hours at 37°C. After one day, the swatches were washed in sterile PBS buffer to release the cell that did not stick to the cotton. Serial dilutions of the washing solution were made. The dilutions were plated on LB medium (10 g/L NaCl, 10 g/L

Bacto-Tryptone and 5 g/L yeast extract) and incubated for 24 hours at 37°C. After incubation, recovered colonies were counted and used to determine percent reductions calculated according to "AATCC Test Method 100-2012" following equation:

$$R = (B-A) / B \times 100\%$$

where: R is the percentage of bacterial reduction; B is the number of bacterial colonies at the initial stage (0 h); and A is the number of bacterial colonies after 24 h of contact with finished substrates.
In the AATCC Test Method 100-2012 method variant, after washing steps, cotton swatches were inoculated in 20 mL of LB medium and incubated at 37°C for 24 hours (220 rpm). After one day the OD_{600} of each conditions were measured.

6.2.6 Antioxidant test
ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 hours before use (Re *et al.* 1999). For the Trolox reaction, the $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.90 at 734 nm. After $ABTS^{\bullet+}$ dilution in 1 mL of reaction, Trolox standards solution were added (final concentration 0–15 μM) and readings were taken after 6 minutes. Appropriate solvent blanks were run in each assay. All determinations were carried out in triplicate. The radical scavenging assay of the treated cotton fiber sample was similar to the above protocol. Smashed fiber sample of four different mass (2–20 mg) were separately reacted with 2 mL of radical solution, the resulting solutions were filtered and measured at corresponding wavelength mentioned above. Radical scavenging activities were calculated according to the equation:

$$\% \text{ Scavenging} = [(Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$$

where $Abs_{control}$ = absorbance of radical solution + untreated cotton and Abs_{sample} = absorbance of radical solution + treated cotton. All measurements were based on triplicate sample. EC_{50} value (µM) was the effective concentration at which the scavenging capacity was 50 % and was obtained by interpolation from linear regression analysis. The EC_{50} values of the fiber samples obtained are presented as µmol equiv Trolox/100 g of fiber (Ma *et al.,* 2016).

6.2.7 Scanning Electron Microscopy (SEM)
The grafted fabrics were dried at 37° for 4 hours and scanned in different places of the sample at 30Kv using an electron microscope model Cambridge 250 Mk3 SEM equipped with a Link System AN10000 energy dispersive spectrometer (EDS) at 12000 magnification. The samples were coated with a thick layer of gold before scanning with SEM and all the images were carefully taken using the same sample position of weft and warp.

6.2.8 Confocal laser scanning microscope visualization of E. coli expressing Green Fluorescent Protein (GFP)
The *E. coli* strain expressing GFP was a gift from Dr. Parrilli E. (University of Naples Federico II, Italy). When the bacterial growth has reached the OD_{600} value of ~0.8 at 37°C, the expression of the fluorescent proteins by *E. coli* transformed with plasmids encoding GFP was induced by addition of 1 mM IPTG. After the IPTG induction the culture was incubated at 25°C for 15 hours (220 rpm). A cell suspension was prepared by diluting the microbial growth in PBS. This suspension was placed onto the top of the swatches (1cm x 1cm) with a concentration of 10^5 CFU/mL. The inoculated swatches were incubated for 24 hours at 37°C. After one day a part of the samples was dropped off on microscope glasses, while the other swatches were shaken for 1 minutes in a sterile PBS buffer and dropped off on microscope glasses. All microscopic observations and image acquisitions were performed with a confocal

laser scanning microscope (CLSM) (LSM700-Zeiss, Germany) equipped with an Ar laser (488nm), and a He-Ne laser (555nm). Images were obtained using a 10X/0.8 objective. The excitation/emission maxima for these dyes are 489/510 nm for eGFP stain and 533/559 nm for 6-Hex. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the swatches. Images were recorded as a series of .jpeg files with a file-depth of 16 bits. For each condition, two independent samples were used.

6.3 Results and discussions

6.3.1 In situ cotton dyeing

A dye based on the POXA1b mediated hetero-coupling of 2,5-dabsa and resorcinol, has been already applied to the dyeing of several fibers (Pezzella *et al.,* 2016). This dye was effective in nylon and wool colorization, while cotton, together with other synthetic fibers, was not colored. As a fact, cotton colorization is a troublesome process requiring high amounts of salt for efficient dye uptake and resulting in the discharge of polluting wastewaters. In this context, the *in situ* colorization of cotton represents an alternative approach to cut on salt consumption and improve dye utilization. Laccases are known for their ability to modify various surfaces through grafting of phenolic moieties, conferring properties such as antimicrobial, hydrophobicity and antioxidant (Piscitelli *et al.,* 2011).

In this work an *in situ* dyeing process was tested for cotton coloration using the enzyme POXA1b and 2,5-dabsa and resorcinol as precursors. This process revealed to be effective in cotton dyeing in comparison with the *ex-situ* one (Fig 1). As a fact, after the incubation of cotton with enzymes and precursors, a deep brown colour was developed on the fiber.

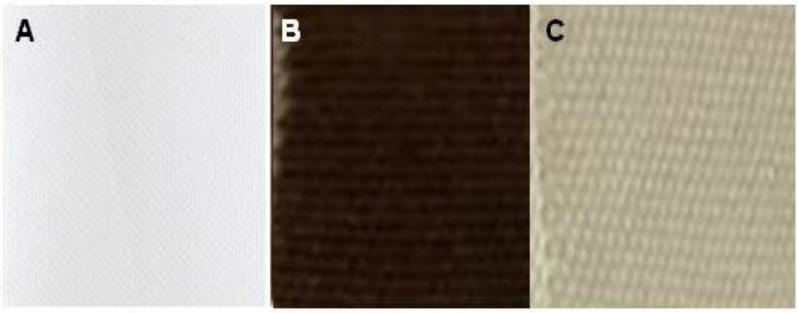

Fig.1 Cotton fibers after 9 hours of incubation: A) cotton fibers before treatment B) cotton fibers with *in situ* dyeing C) cotton fiber with *ex situ* dyeing.

Fiber colorization could be the result of covalent bond or strong electrostatic interaction of sulphonic groups to the cotton. To verify the nature of the interaction between cotton and the dye, an ATR-FTIR analysis has been performed. However, due to the low concentration of the dye on the fabric and overlapping with characteristic absorbance of cellulose, a well-defined absorption band for the colorant was difficult to detect (data not shown).

In order to evaluate the potential damaging caused by the grafting reaction to the fiber, the functionalized samples were analyzed by means of scanning electron microscopy (SEM). Microphotographs clearly showed that the colored fibers were not damaged. The textile morphology remained regular in the presence of the dye (Fig. 2B) treatment compared with the untreated control (Fig. 2A).

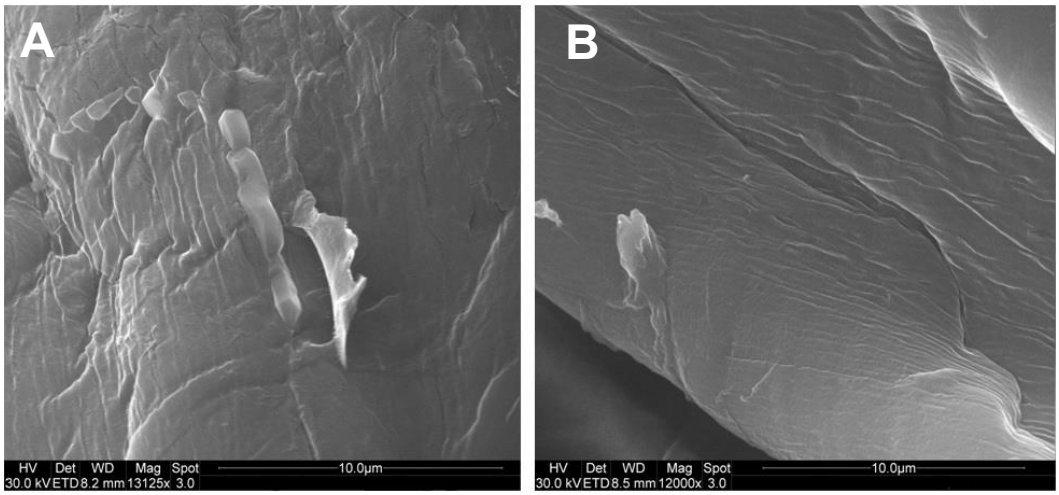

Fig. 3. Scanning electron microscopy images of cotton fabrics: A) cotton fibers before treatment B) Functionalized fibers. All the images were amplified 12000.

Once the coloring properties were assessed, an investigation about other potential properties (hydrophobicity, antioxidant and antimicrobial activity) conferred to the surface was carried out. As far as hydrophobicity, no variation in wettability of the treated sample was observed.

6.3.2 Antioxidant activity

There are several studies about antioxidant activity that consider resorcinol derivatives and other polyphenols to be radical scavengers (Velika *et al.,* 2013, Choi *et al.,* 2012 and Bendary *et al.*, 2013). The radical scavenging activity of the treated cotton fibers was evaluated.

Tab. 1 ABTS cation scavenging activities of treated and no treated cotton fiber samples

	% Scavenging against ABTS	[a]ABTS radical scavenging activity EC_{50}
Treated cotton	96.2±4.2	17.4
No treated cotton	4.3±1.5	-

[a] Expressed as µM equiv Trolox/mg of fiber sample

As showed in Table 1, the treated cotton fiber showed a percentage of scavenging activity values of about 96%. The slight percentage observed for the untreated cotton was probably due to the absorbance of $ABTS^{\bullet+}$ to the fiber. Thus, the *in situ* dyeing process also conferred to the cotton fiber a powerful radical scavenger activity.

6.3.3 Anti-microbial activity

Antibacterial activity of treated cotton was tested against an example of Gram positive (*B. subtilis*) and Gram negative (*E. coli*) bacteria using the standard ‘‘AATCC Test Method 100-2012". Different controls were set up in order to understand the role played by laccase and both precursors in conferring the target property.

It is worth to note that the *in situ* dyeing process did not confer any antimicrobial activity to the textile (Fig. 4). Conversely, textiles endowed with a significant antimicrobial activity towards *B. subtilis* were obtained in the presence of resorcinol,

with an enhanced effect in the laccase promoted grafting process. The activity observed in the absence of enzyme may be due to resorcinol self-oxidation at the reaction conditions (pH 8 and 60°C) and is in line with already reported examples (Paidesetty *et al.,* 2015). 2,5-dabsa did not exert any antimicrobial effect, either in the presence or in the absence of laccase. Taken together, these results indicated that in the presence of the enzyme a functionally different dyeing molecule is obtained on the fiber, suggesting the occurrence of a product structurally different from that obtained from the spontaneous oxidation of resorcinol and 2,5-dabsa.

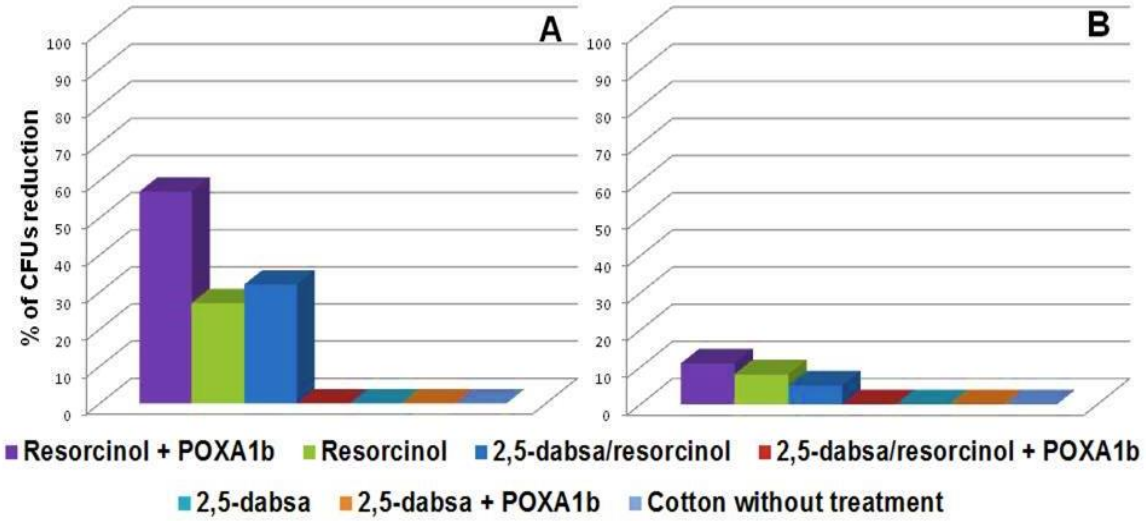

Fig. 4. Antibacterial activity of different cotton samples evaluated according to the AATCC Test Method 100-2012 towards (A) *B. subtilis* and (B) *E. coli*. Standard deviations among three replicates were less than 5%.

6.3.4 Anti-adhesive activity

A variation to the ATCC Test Method 100-2012 for antimicrobial activity was introduced to get insight into the bacteria adhesion onto cotton fiber. As a fact, after incubation with the tested bacteria species, the swatches were washed in sterile water and then inoculated in LB liquid medium. Data in Fig 5 reported the microbial growth resulting from the inoculation of cotton swatches following this washing step.

Results showed that after inoculation of the cotton swatches functionalized with 2,5-dabsa/resorcinol in the presence of POXA1b, no microbial growth was observed, indicating an anti-adhesion effect towards both bacteria tested. Also the swatches treated with resorcinol, with and without laccase, caused a reduction in the microbial growth with respect to the untreated cotton. However, this result is explainable considering the antimicrobial effect previously demonstrated for cotton functionalized with only resorcinol.

It can be concluded that cotton functionalized through the *in situ* dyeing reaction affected bacterial adhesion allowing effective cell removal after a simple washing step in PBS buffer. The untreated cotton, instead, is still able to promote microbial growth when inoculated, even after this washing step.

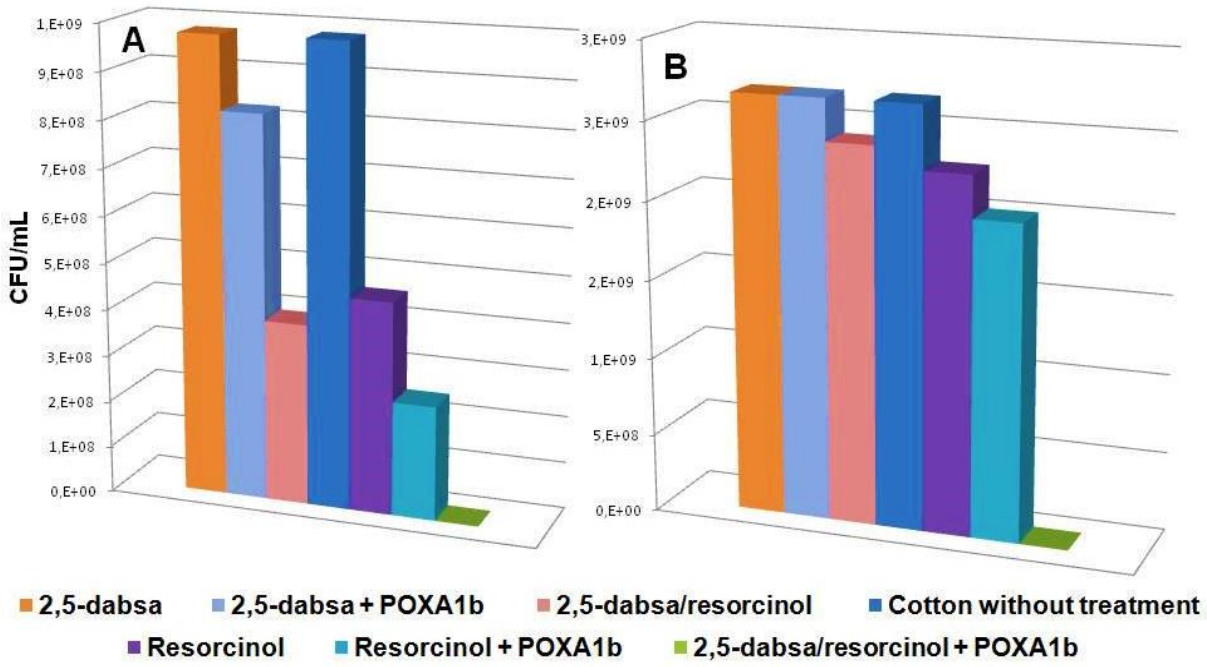

Fig. 5. Microbial growth of (A) *B. subtilis* and (B) *E. coli* after 24 hours of incubation with different cotton swatches. Standard deviations among three replicates were less than 5%.

A Confocal laser scanning microscope visualization experiment was developed in order to confirm that the functionalized cotton fiber has acquired an anti-adhesion property after the enzymatic treatment. First experiments were carried out visualizing the textile after cell staining with DAPI (4′,6-diamidino-2-phenylindole). Unfortunately the treated fiber showed an intrinsic auto-fluorescence and cross-reacted with the fluorescent dye (data not shown). For this reason, a *E. coli* strain expressing a Green Fluorescent Protein (GFP) was used. Unfortunately, the functionalized fibers was excited at the same wavelength of GFP fluorophore. In order to distinguished the fibers from the bacteria, the functionalized cotton was excited with two different wavelengths corresponding at two diverse chromophores molecules, GFP and 6-Hex (Fig. 6). The latter was chosen to excite the fiber itself to better distinguish cell fluorescence from the background.

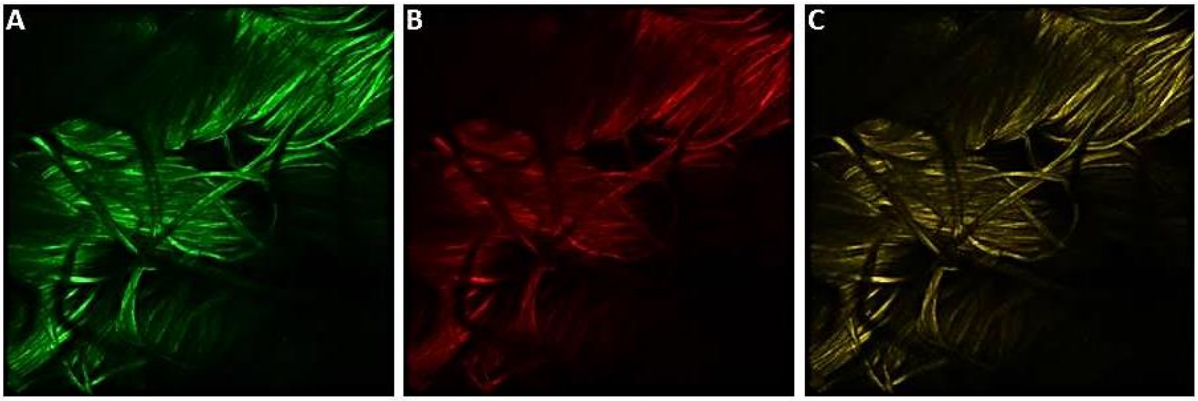

Fig. 6 Functionalized cotton fiber visualized by Confocal laser scanning microscope. A) exited at GFP absorbtion wavelength; B) exited at 6-Hex absorption wavelength; C) exited by GFP and 6-Hex wavelength at the same time.

After the setting up of the confocal parameters, the swatches obtained “before” and “after” the washing step of the ‘‘AATCC Test Method 100-2012 were analyzed (Fig. 7).

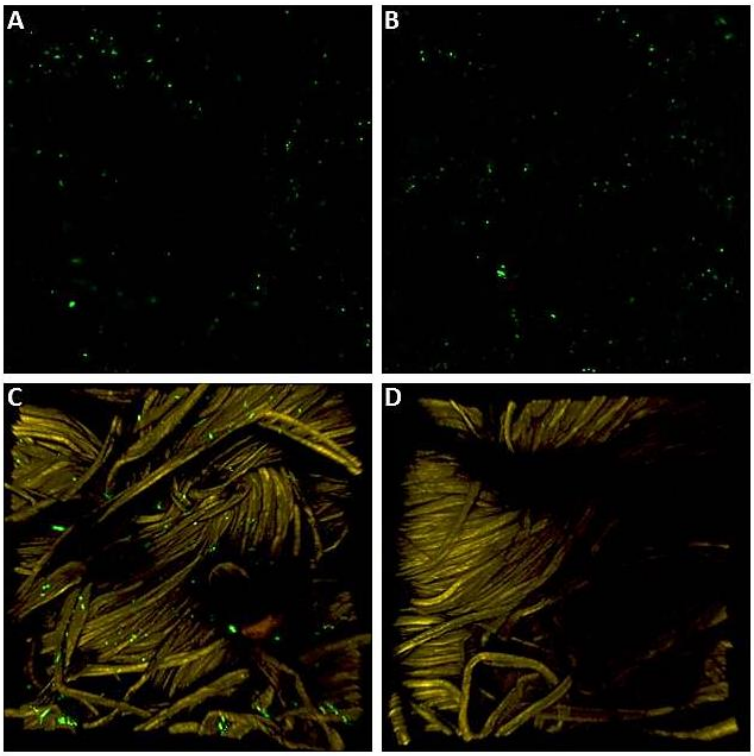

Fig. 7 Cotton fiber visualized by Confocal laser scanning microscope. A) cotton fibers before washing; B) cotton fibers after washing; C) Functionalized cotton fibers before washing; D) Functionalized cotton fibers after washing.

As expected, the cells after washing were completely removed from the functionalized textile, while they persisted after the cleaning step on untreated cotton fibers, confirming the anti-adhesive property of the coating swatches.
B. subtilis and *E. coli* were reported to exhibit a superficial negative charge due to the presence of teichoic acids linked to the peptidoglycan and to the outer covering of phospholipids and lipopolysaccharides, respectively, (Dickson *et al.,* 1989).
The pKa of the synthesized molecule was estimated through computational analysis. A putative structure of the product synthesized on the fiber was hyphotesized and analyzed with the Epoch software (https://epoch.uky.edu/ace/public/pKa.jsp). This *in silico* analysis has estimated the pKa of each atom of the reaction product (Fig. 8). As expected the oxygen atom bonded to sulfonic group of the polymer had a pKa of 2.3, suggesting that at the pH used for the experimental tests (pH ~7) the polymer is negatively charged.

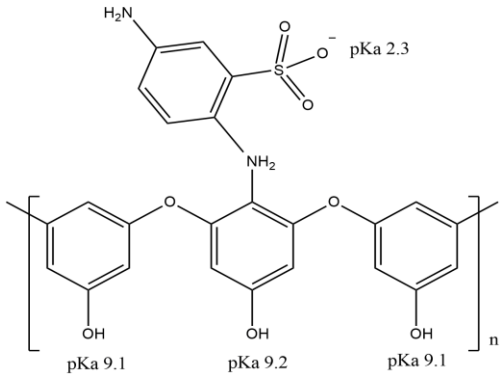

Fig. 8 pKa values of the hetero-polymer

The anti-adhesive effect could thus be due to repulsion interactions between the negative charge of the bacterial surface and negative one of the grafted dyeing molecule.

6.3.5 Washing test

The durability of the finishing applied on the textile material after washing is one of the main concern of today's textile industry. For this reason, the cotton swatches were washed simulating a classical laundry process and then their anti-adhesive properties were tested according to previously adopted ATCC test variant. Also in this case, the test microorganism was *E. coli* expressing GFP and the swatches were compared before and after the washing step including in ''AATCC Test Method 100-2012" (Fig. 9).

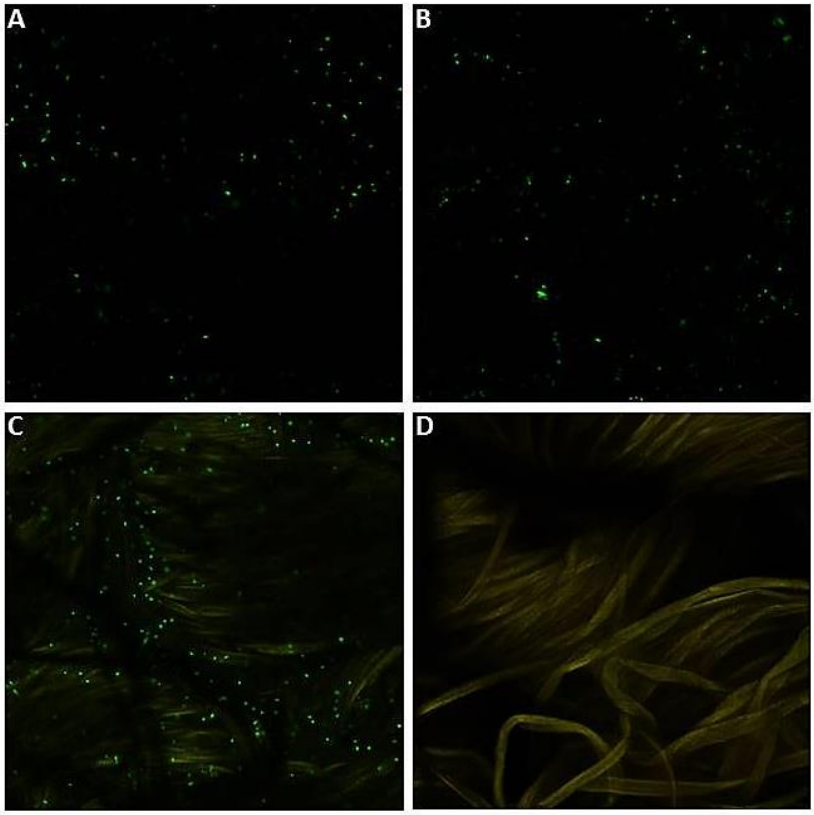

Fig. 9 Washed cotton fiber visualized by Confocal laser scanning microscope. A) cotton fibers before washing; B) cotton fibers after washing; C) Functionalized cotton fibers before washing; D) Functionalized cotton fibers after washing.

Also in this case the results indicated that the sample after laundry process did not lose its anti-adhesion property, avoiding completely the cellular adhesion.

6.4 Conclusions

In this work an *in situ* dyeing process was tested for cotton coloration using the enzyme POXA1b and 2,5-dabsa and resorcinol as precursors. The process was found to be effective in fiber dyeing. In addition, the functionalized textile acquired anti-adhesive properties towards *E. coli* and *B. subtilis* as well as interesting antioxidant activity.
The modified textile preserved its qualities also after washing. Further studies like bioprocess optimization, cost evaluation, test on pathogenic strains, preclinical studies will be needed for the effective exploitation of this multifunctional textile in home furnishings, clothing and medical textile.

6.5 References

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Conclusions

Conclusions

In conclusion, the present PhD project has successfully reached the goal to transfer the innovative research results from Academia to Industry. In order to reach this aim the project was divided in two phases:

1. developing and improving laccase POXA1b
2. developing of innovative and eco-sustainable industrial processes.

As for the first topic, POXA1b laccase was heterologously expressed and an economic analysis of its recombinant production process has been performed. In fact, the high cost of laccases production represents the principal bottleneck step of their commercialization. For this reason, the POXA1b low price obtained has demonstrated that the production process can be exploitable at industrial scale.
Moreover, a POXA1b immobilization process was optimized by statistic method to increment the operational stability of the enzyme in specific processes.
Furthermore, this thesis showed that laccase active site is a promiscuous template to increase activity toward specific target substrates, through the design of stabilizing new enzyme–substrate interactions.
As regarding the industrial applications, the recombinant enzyme POXA1b has successfully replaced the traditional chemical methods in different industrial fields such as dye, textile and beverage industries, expanding its panel of applicability.

Publications and communications

PUBLICATION:

1. **Giacobelli V.G.** and Monza E., Piscitelli A., Pezzella C., Lucas F., Guallar V. and G. Sannia. Repurposing designed mutants: a valuable strategy for computer-aided laccases engineering. The case of POXA1b. Catal. Sci. Technol., 2017,7, 515-523.

2. Pezzella C., Giacobbe S**, Giacobelli V. G.,** Guarino L., Kylic S., Sener M., Sannia G, Piscitelli A.. Green routes towards industrial textile dyeing: A laccase based approach. J. Mol. Catal. B: Enzym. 2016, 134 (2016) 274–279.

3. Lettera V., Pezzella C., Cicatiello P., Piscitelli A., **Giacobelli V. G.,** Galano E., Amoresano A. and Sannia G. Efficient immobilization of a fungal laccase and its exploitation in fruit juice clarification. Food Chemistry Volume 196, 1 April 2016, Pages 1272–1278.

COMMUNICATION:

POSTER

1. **Giacobelli V.G.,** Monza E., Piscitelli A., Pezzella C., Lucas F., Guallar V. and G. Sannia. Engineering laccase POXA1B through computational methods. 8th European Meeting on oxidative enzymes (OxiZymes) 3–6 July 2016, Wageningen, Netherlands

2. **Giacobelli V. G.,** Pezzella C., Piscitelli A., Cicatiello P., Sannia G. Pichia Pastoris heterologous expression: filling the GAP...promoter with glycerol. 8th Conference on Recombinant Protein Production (RPP) 22-24 April 2015, Palma de Mallorca, Spain

ORAL PRESENTATION

1. **Giacobelli V.G.,** Piscitelli A., Pezzella C., Guarino L., Kilic S., Giacobbe S., Sener M., Sannia G. Laccase assisted synthesis of a new eco-friendly polymeric dye. 9th International Conference on Fiber and Polymer Biotechnology (IFBP) 7-9 September 2016, Osaka, Japan

RESEARCH ACTIVITY IN FOREIGN LABORATORY

From April 1st to August 31th my research activity was carried out in Prof. Victor Guallar's laboratory at Barcelona Supercomputing Center, Computational Biology Research Center, Barcelona, Spain.