RECOMBINANT EXPRESSION OF FUNGAL OXIDASES FOR INDUSTRIAL APPLICATION

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Al mio Sposo

SUMMARY

Laccases catalyse the oxidation of a range of organic substrates coupled to the reduction of molecular oxygen to water. They are members of the ubiquitous blue multi-copper oxidase family. These enzymes are implicated in a wide variety of biological activities. Most of the laccases studied thus far are of fungal origin. Large variety of potential substrates has raised interest in the use of laccases in several industrial applications, such as pulp delignification, textile dye bleaching, effluent detoxification, biopolymer modification and bioremediation. Cloning of the laccase genes followed by heterologous expression may provide higher enzyme yields and may permit to produce laccases with desired properties (different substrate specificities and improved stabilities) for industrial applications.

Heterologous expression of *Pleurotus ostreatus* laccases POXC and POXA1b in two yeasts and a first approach of directed evolution experiments are reported. The yeasts of choice were *Saccharomyces cerevisiae*, proven to be success-full in recombinant laccase expression and directed-evolution experiments, and *Kluyveromyces lactis*, a non-conventional yeast offering significant advantages, such as high-level secretion of non-hyperglycosylated recombinant proteins. Expression vectors were set up cloning the cDNAs under the control of different promoters. Furthermore, the laccase leader peptides (*poxc* and *poxa1b*), as well as the yeast derived signal peptides (*S. cerevisiae* invertase and *K. lactis* killer toxin), were alternatively used to direct the secretion of active laccase into the culture medium. The laccase signals proved to be more effective to drive the secretion of recombinant proteins in both hosts.

Levels of laccase secreted activity were markedly different: rPOXA1b transformants always gave much higher activity than rPOXC transformants, and production of both laccases in *S. cerevisiae* was significantly lower than that in *K. lactis*.

Recombinant laccases from *K. lactis* were purified to electrophoretic homogeneity and characterized. rPOXA1b specific activity was similar to that of the native protein, whilst rPOXC specific activity was much lower than that of the native POXC. Mass spectrometry analyses of the recombinant proteins allowed to verify their primary structures and to identify post-translational modifications. Our data confirm that *K. lactis* has a lower tendency, respect to *S. cerevisiae*, to hyperglycosylate recombinant proteins. The *S. cerevisiae* laccase expression systems were further used to set off directed evolution experiments. Mutated cDNAs libraries with different mutation rate were created, and homologous recombination experiments were performed, giving rise to libraries of mutated laccase secreting yeasts. Moreover a screening procedure to isolate clones exhibiting desired property was realized.

As a result, this work allowed obtaining the heterologous expression of two *P. ostreatus* laccases in yeasts, and their purification and characterisation. Moreover, this research work broadened the potentiality of the developed expression system addressing enzymes to such large markets and different industrial application such as pulp and textile bleaching, and enzymatic remediation of waste streams. A new laccase host (*K. lactis*) has been built on, and its promising performances will lead to further investigate its utilization for further structure-activities studies, as well as for directed evolution. Results obtained demonstrate the potential of the recombinant expression for the study of potential industrial interest.

SOMMARIO

Le fenolo-ossidasi, cupro-proteine che catalizzano l'ossidazione di *p*-difenoli con la concomitante riduzione di ossigeno molecolare ad acqua, sono enzimi variamente distribuiti in natura: attività laccasica è stata ritrovata in più di 60 ceppi di funghi appartenenti a varie classi, e in alcune piante superiori; la presenza di enzimi "laccasi-like" è stata, inoltre, riportata in batteri e in insetti. Negli ultimi anni la ricerca intorno a questa classe di enzimi è stata molto intensa, legittimata soprattutto dal fatto che, tra i vari agenti biologici, le laccasi rappresentano un interessante gruppo di enzimi ossidoreduttivi ubiquitari, molto promettenti per una serie di applicazioni biotecnologiche. Commercialmente, esse sono state utilizzate nella demolizione di materiali ligninolitici, nella produzione di etanolo, e nel processo di "scolorimento" dei jeans DENIM. Inoltre, una gran varietà di processi di *bioremediation* impiega laccasi al fine di preservare l'ambiente dai danni causati dagli effluenti industriali. L'applicazione reale ed effettiva delle laccasi passa, necessariamente, attraverso la loro sempre più completa caratterizzazione, e, proprio nel tentativo di comprendere meglio il ruolo funzionale delle laccasi (soprattutto fungine), la ricerca intorno a questa classe di enzimi è stata, negli ultimi anni, molto intensa. Di qui il notevole interesse nella messa a punto di sistemi di espressione ricombinante. La messa a punto di un sistema di espressione ha, poi, un ulteriore vantaggio, ancora più avvincente, quello cioè di poter creare enzimi su misura (stabilità, specificità, velocità di reazione, etc.) per differenti applicazioni.

Da diversi anni i lieviti sono divenuti organismi di eccellenza per l'espressione di proteine eterologhe: *Pichia pastoris*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Schizosaccaromyces pombe*, *Saccharomyces cerevisiae* sono stati particolarmente studiati ed adattati per questo fine. Essi costituiscono sistemi attraenti in vari aspetti della moderna biotecnologia, in quanto sono microrganismi unicellulari eucarioti che combinano la semplicità delle manipolazioni genetiche con la capacità di realizzare modifiche post-traduzionali sui polipeptidi espressi.

Tale lavoro di ricerca si è focalizzato sull'espressione ricombinante e sull'evoluzione guidata di laccasi dal fungo basidiomicete *white-rot Pleurotus ostreatus*. Come la maggior parte delle specie fungine, *P. ostreatus* produce vari isoenzimi laccasici. Ben cinque isoenzimi fenolo ossidasici (POX-: Phenol OXidase-) sono stati isolati e caratterizzati: POXC, POXA1b, POXA1w, POXA3a e POXA3b. Tra questi, sono stati scelti come "modello" nella messa a punto e sviluppo di sistemi di espressione di laccasi in lieviti, due particolari isoenzimi: POXC, l'isoenzima più abbondante in tutte le condizioni di crescita fino ad ora esaminate; e POXA1b, enzima particolarmente stabile a pH alcalini. Tale caratteristica risulta molto interessante per una sua applicazione nello smaltimento dei reflui industriali, spesso caratterizzati da un pH fortemente alcalino, oltre che da concentrazioni consistenti di fenoli. I lieviti scelti per realizzare tali sistemi di espressione sono *S. cerevisiae* e *K. lactis*. Da anni, ormai, *S. cerevisiae* è utilizzato come ospite per l'espressione eterologa di proteine: la sua genetica è ampiamente conosciuta e caratterizzata, si ha a disposizione un buon numero di vettori per l'espressione, ed inoltre è già stato utilizzato, e con successo, nella produzione di laccasi ricombinanti. Negli ultimi anni, comunque, un crescente numero di lieviti non-*Saccharomyces* sta divenendo accessibile, grazie alle moderne tecniche della genetica molecolare: alcuni di essi offrono caratteristiche favorevoli, come alti livelli di secrezione, disponibilità di sequenze promotrici molto forti o strettamente regolate. *K. lactis*,

organismo non patogeno dotato di buone capacità di secrezione, ed appartenente alla classe dei lieviti "non convenzionali", sembra ben sostituire *S. cerevisiae.* I tentativi di impiego di tale lievito su larga scala per la produzione di proteine ricombinanti risultano relativamente recenti, ed il particolare interesse sembra derivare dalle sue peculiari caratteristiche, quali, ad esempio, la sua abilità di processare e secernere proteine eterologhe ad alto peso molecolare in modo molto simile a quello degli eucarioti superiori, e di produrre proteine non iperglicosilate.

Per l'espressione eterologa delle laccasi POXC e POXA1b sono stati realizzati vari costrutti utilizzando differenti promotori- sia inducibili che costitutivi- deputati all'attivazione trascrizionale del gene di interesse. Inoltre, per la secrezione delle proteine nel mezzo di coltura, sono state utilizzate sia sequenze segnale di lievito (invertasi da *S. cerevisiae*, e tossina killer da *K. lactis*) che sequenze proprie delle laccasi, confrontando la loro efficienza nel dirigere la secrezione. Per entrambe le laccasi, ed in entrambi i lieviti, l'utilizzo del peptide segnale omologo alle laccasi ha consentito una maggiore secrezione di attività laccasica.

La produzione di proteina ricombinante è stata ottimizzata variando le condizioni di crescita: tali esperimenti hanno anche permesso di valutare l'influenza della concentrazione di induttore, della composizione del terreno di coltura, e della concentrazione di rame (cofattore delle laccasi), sulla produzione stessa. Entrambi i lieviti si sono rivelati capaci di secernere i due isoenzimi laccasici nel mezzo di coltura. La quantità di attività laccasica secreta è risultata, però, significativamente differente per i due isoenzimi espressi: in particolare, in tutte le condizioni di crescita esaminate, i cloni trasformati con i vettori contenenti il cDNA di *poxa1b* hanno consentito una maggiore secrezione di attività laccasica dei cloni trasformati con i vettori contenenti il cDNA di *poxc*. È stata, inoltre, osservata una significativa differenza nella produzione tra i due diversi ospiti: *K. lactis* è in grado di secernere laccasi ricombinanti in misura molto maggiore rispetto a *S cerevisiae*. La migliore produzione di laccasi ricombinanti secrete ottenuta nei due ospiti è di seguito schematizzata:

rPOXA1b K. lactis	rPOXC K. lactis	rPOXA1b S. cerevisiae	rPOXC S. cerevisiae
4.2 U ml^{-1} (3 giorni)	0.09 U ml^{-1} (4 giorni)	0.2 U ml^{-1} (7 giorni)	$8.4*10^{-4}$ U ml^{-1} (4 giorni)

Inoltre, in *K. lactis* le laccasi ricombinanti prodotte sono equamente distribuite tra cellule e mezzo extra-cellulare, mentre in *S. cerevisiae* sono, per la maggior parte, ritenute all'interno delle cellule. Esperimenti di immunorivelazione hanno, poi, confermato la tendenza di *S. cerevisiae* ad iperglicosilare le proteine ricombinanti: la frazione secreta di rPOXA1b presenta un peso molecolare apparente molto maggiore sia della proteina nativa che della frazione intracellulare della proteina ricombinante, mentre non si apprezza una grande differenza tra le frazioni secreta ed intracellulare di rPOXC. Al contrario, in *K. Lactis*, il peso molecolare apparente della frazione secreta di rPOXA1b, pur essendo maggiore di quello della proteina nativa, si differenzia da questa in misura

meno significativa. I risultati ottenuti confermano quindi che *K. lactis* è dotato di un buon sistema di secrezione, e che la sua tendenza ad iperglicosilare le proteine ricombinanti è meno marcata che in *S. cerevisiae*. Da un'attenta analisi delle curve di produzione e degli esperimenti di immunorivelazione, sembra che l'inefficiente secrezione sia uno dei fattori che potrebbe spiegare la differente produzione di attività laccasica tra i due lieviti. L'inefficiente secrezione non sembra, però, spiegare la differente produzione tra i due isoenzimi, ed altre potrebbero essere le cause implicate nella scarsa attività secreta di rPOXC nei due organismi ospiti.

Le laccasi ricombinanti espresse in *K. lactis* sono state purificate all'omogeneità, caratterizzate, e confrontate con le corrispondenti proteine estrattive. Se si considera la quantità di proteina ricombinante purificata, e la resa del processo di purificazione, la produzione è paragonabile per entrambe le proteine (1.4 e 1.1 mg l^{-1}, rispettivamente per rPOXC e rPOXA1b).

Esperimenti condotti mediante spettrometria di massa MALDI hanno consentito di validare le sequenze delle due laccasi (83% per r-POXA1b e 70% per rPOXC) e di determinare i siti glicosilati in *K. lactis*. Tali esperimenti hanno anche consentito l'identificazione di una maturazione al C-terminale per rPOXA1b, maturazione che, per altro, era stata già identificata nella proteina nativa. rPOXA1b è stata trovata depleta degli ultimi due amino acidi, mentre una certa eterogeneità era stata riscontrata al C-terminale della proteina nativa. Tale *processing* suggerisce la presenza, nella sequenza di POXA1b, di un segnale riconosciuto sia dalle proteasi di *P. ostreatus* che da quelle di *K. lactis*. Ulteriori indagini saranno comunque necessarie per identificare tale segnale, e per valutare un eventuale ruolo funzionale della maturazione del C-terminale.

L'attività specifica di rPOXC è molto inferiore (circa due ordini di grandezza) di quella della proteina nativa, mentre non si riscontrano particolari differenze tra l'attività specifica di rPOXA1b e quella della corrispondente proteina nativa. Per quel che riguarda, invece, l'affinità nei confronti dei due substrati utilizzati, questa risulta essere paragonabile o maggiore per le laccasi ricombinanti. Anche l'enzima rPOXA1b da *S. cerevisiae*, la cui affinità per i due substrati è stata determinata, esplica un comportamento catalitico simile o migliore di quello della proteina nativa.

Una volta messo a punto il sistema di espressione in *S. cerevisiae*, tale lavoro di ricerca si è avvalso di una moderna scoperta della biotecnologia, l'evoluzione guidata mediante mutazione casuale. Infatti, la facilità di manipolazione di *S. cerevisiae*, insieme all'alta frequenza di ricombinazione, e ai recenti successi nell'evoluzione guidata di laccasi, rendono questo lievito il più utilizzato nella realizzazione di questo tipo di esperimenti. Per la mutagenesi casuale è stato utilizzato il vettore che ha consentito la produzione maggiore di proteina ricombinante, B-pSAL4, creando, quindi, enzimi rPOXA1b* con differenti proprietà catalitiche e/o chimico fisiche. Sono stati realizzati esperimenti di polimerizzazione a catena in condizioni di reazione tali da avere un basso ed un medio tasso di mutazioni (0-3 e 3-7 mutazioni/Kb); la collezione di cDNA variamente mutati così costruita è stata inserita nei vettori di espressione mediante esperimenti di ricombinazione omologa *in vivo*; la collezione di tali vettori di espressione è stata trasformata nel lievito ed analizzata su terreno selettivo. Sono stati creati centinaia di ceppi producenti proteine variamente mutate. Infine, è stata messo a punto una metodologia di *screening* per la selezione di questi mutanti, al fine di evidenziare

proteine che esplichino particolari proprietà, come ad esempio differente stabilità al pH o alla temperatura, differente specificità di substrato, etc.

In definitiva, questo lavoro ha consentito lo sviluppo e la messa a punto di sistemi di espressione di due laccasi in lieviti, e la costruzione di enzimi *ad hoc* per specifiche applicazioni industriali. Il sistema di espressione in *K. lactis* consente la produzione di discrete quantità di laccasi ricombinanti, di cui si è proceduto alla purificazione e ad un'ampia caratterizzazione. Il sistema di espressione in *S. cerevisiae*, oltre che produrre laccasi ricombinanti, pur se in quantità minori, ha consentito la creazione di enzimi mutati. I risultati ottenuti dimostrano l'enorme potenziale dei sistemi di espressione ricombinanti di laccasi, ponendo le basi per studi futuri. Si può affermare, infatti, che è stato identificato un nuovo ospite (*K. lactis*) per l'espressione ricombinante di laccasi, le cui positive caratteristiche e prestazioni spingono ad ulteriori indagini per ampliarne le applicazioni, anche nel campo dell'evoluzione guidata.

RESUMÉ

Les laccases catalysent l'oxydation d'une grande variété de substrats organiques couplée à la réduction de l'oxygène moléculaire en eau. Elles font partie d'une famille d protéines ubiquitaires: les oxydases bleues multicuivres. Ces enzymes sont impliquées dans de nombreuses réactions biologiques. La plupart des laccases étudiées jusqu'à présent sont d'origine fongique. La grande variété de substrats potentiels des laccases a suscité un grand intérêt pour l'utilisation des laccases dans différentes applications industrielles, parmi lesquelles je citerai la délignification, la décoloration des textiles, la détoxification d'effluents, la modification de biopolymères et la biorémédiation. Le clonage de gènes codant pour des laccases suivi de leur expression hétérologue peut permettre d'obtenir de grandes quantités d'enzymes et peut permettre de produire des laccases ayant des propriétés choisies (ayant des spécificités de substrats, une stabilité améliorée par exemple) pour des applications industrielles.

Nous décrivons dans ce mémoire l'expression hétérologue des laccases POXC et POXA1b provenant du champignon *Pleurotus ostreatus* dans deux levures différentes ainsi qu'une première approche d'expériences d'évolution dirigée des laccases. Les levures utilisées sont *Saccharomyces cerevisiae*, dont la capacité à produire des laccases et l'efficacité en tant qu'outil dans les expériences d'évolution dirigée sont connues, et *Kluyveromyces lactis*, une levure non conventionnelle offrant de nombreux avantages tel qu'un fort niveau de sécrétion de protéines recombinantes qui ne sont pas hyperglycosylées.

Nous avons construit des vecteurs d'expression en clonant les ADNc sous le contrôle de différents promoteurs. De plus, nous avons utilisé alternativement les séquences d'adressage natives des laccases POXC et POXA1b et des séquences d'adressages de levures (de l'invertase de *S. cerevisiae* ou de la toxine létale de *K. lactis*) pour diriger la sécrétion de laccases actives dans le milieu de culture. Nous montrons que les séquences d'adressage natives sont plus efficaces que les séquences de levures pour diriger la sécrétion des protéines recombinantes et ce dans les deux hôtes utilisés.

Les niveaux d'activité détectés pour les deux protéines recombinantes sécrétées sont très différents: les transformants produisant rPOXA1b ont toujours une activité apparente beaucoup plus importante que les transformants produisant rPOXC. Nous observons de plus que la production des deux protéines recombinantes est beaucoup plus faible chez *S. cerevisiae* que chez *K. lactis*.

rPOXA1b K. lactis	rPOXC K. lactis	rPOXA1b S. cerevisiae	rPOXC S. cerevisiae
4.2 U ml^{-1} (3 jours)	0.09 U ml^{-1} (4 jours)	0.2 U ml^{-1} (7 jours)	$8.4*10^{-4}$ U ml^{-1} (4 jours)

Nous avons purifié les protéines recombinantes produites chez *K. lactis* jusqu'à l'homogénéité et nous les avons caractérisées. L'activité spécifique de rPOXA1B est similaire à celle de la protéine native. En revanche, l'activité spécifique de rPOXC est

beaucoup plus faible que son équivalent purifiée à partir du champignon *P. ostreatus*. Les analyses en spectrométrie de masse des protéines recombinantes ont permis de vérifier leur structure primaire et l'état des modifications post-traductionnelles. Nos résultats montrent que, par rapport à *S. cerevisiae*, *K. lactis* a une tendance moindre à hyperglycosyler les protéines recombinantes.

Le système d'expression hétérologue dans la levure *S. cerevisiae* a été utilisé pour mettre en œuvre les techniques d'évolution dirigées. Des banques d'ADNc mutés ont été crées avec différents taux de mutations et des collections de levures sécrétants des laccases mutées ont été obtenues par recombinaison homologue. De plus, un procédé de criblage pour les propriétés recherchées a été établi.

Ce travail nous a permis de réaliser l'expression hétérologue de laccases dans deux levures différentes, de purifier les protéines recombinantes et de les caractériser. Les résultats obtenus permettent d'élargir les potentialités des systèmes d'expression hétérologues vers de larges marchés et des applications industrielles telles que la décoloration de la pâte à papier et des textiles et la rémédiation d'effluents.

Un nouveau système à été construit pour l'expression des laccases et ses performances prometteuse conduisent à poursuivre les recherches sur son utilisation dans des procédés industriels ainsi que pour l'évolution dirigée. Les résultats obtenus démontrent, s'il en était encore besoin, le potentiel de l'expression de protéines recombinantes.

INTRODUCTION

Agenda 21, the work programme adopted by the 1992 United Nations Conference on Environment and Development, asserted that biotechnology "promises to make a significant contribution in enabling the development of, for example, better health care, enhanced food security through sustainable agricultural practices, improved supplies of potable water, more efficient industrial development processes for transforming raw materials, support for sustainable methods of aforestation and reforestation, and detoxification of hazardous wastes."

Retting of flax was the first biotechnological application in textile processing. More than 2000 years ago microorganisms grown on flax were used to achieve partial decortication in the extraction of linen fibres from flax stems. One of the earliest examples of industrial enzymes use was the production of whiskey. Over the years, enzymes have also been used in beauty and oral care products, textiles, food and for the fermentation of cheese, beer and wine.

The last century saw the replacement of plant-derived products with petroleum derivatives. These remarkable transformations helped humanity to overcome some of the natural limitations of relying on natural processes. The change was largely a result of advances in chemistry and allied fields. This century promises to open new avenues for increasing the use of renewable resources in the global economy, and, although the future is unpredictable, it is highly likely that biotechnology will play a visible and significant role (more than it did in the 20th century).

Nowadays, industrial biotechnology covers two distinct areas. The first area is the use of renewable raw materials (biomass) to replace raw material derived from fossil fuels. The second is the use of biological systems such as cells or enzymes (used as reagents or catalysts) to replace conventional, non-biological methods.

Enzymes catalyse chemical reactions with great specificity and rate enhancements, as these reactions are the basis of the metabolism of all living organisms. As a result, they offer great potential for cleaner industrial production, and provide tremendous opportunities for industry to carry out elegant, efficient and economical biocatalytic conversions. In other words, biocatalysts generate few by-products and can start with relatively less purified feedstocks. The food, feed, agriculture, paper, leather and textile industries are well suited for enzyme technology because products as well as raw materials consist of (bio) molecules, which can be produced, degraded or modified by enzymatic processes. Many enzymes are commercially available, and numerous industrial applications have been described (Table 1) (Kirk *et al.*, 2002). Over 500 products covering 50 applications, from detergents to beer making, utilize enzymes produced through large-scale fermentation of microorganisms. The list of enzyme applications grows, bringing with it a list of significant social and environmental benefits. The estimated worldwide industrial enzyme market in 2000 was approximately $1.5 billion (of which North America and Europe accounted for 35% and 31% of, respectively) and is traditionally divided into three segments. The largest, at 65% of sales, is that of technical enzymes, the second largest segment, at 25% of the market, is that of food enzymes, finally, feed enzymes contributes approximately to 10% of the market.

Table 1 Enzymes used in various industrial segments and their application.

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, colour 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), flavour
	Lipase	Cheese flavour
	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
	β-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavour (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

Although the advantages of replacing chemical treatments with enzymes are often compelling from a societal and environmental viewpoint, enzymes must compete economically with often entrenched and extremely inexpensive traditional chemical processes. The reduced enzyme prices may dramatically increase the number of applications and even enable large scale processes. Commercial enzyme products were originally developed as simple fermentation broths of naturally occurring organisms. Often the resulting products were complex mixtures of secreted enzymes produced at relatively low yields (Cherry *et al.*, 2003). Today, over 90% of industrial enzymes are produced recombinantly to maximize product purity and economy of production. Microorganisms such as moulds, yeasts or bacteria, are being improved to work as "cell factories" to efficiently produce industrial enzymes targeted at specific tasks. Furthermore, the latest developments within modern biotechnology, introducing protein engineering and directed evolution, have further revolutionized the development of industrial enzymes. These advances have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions, enabling a further expansion of their industrial use.

A versatile enzyme with a relative small market is laccase. An application in textile wet processing and the manufacturing of Denim is shown in figure 1. Laccases market might grow rapidly if their performance can be improved.

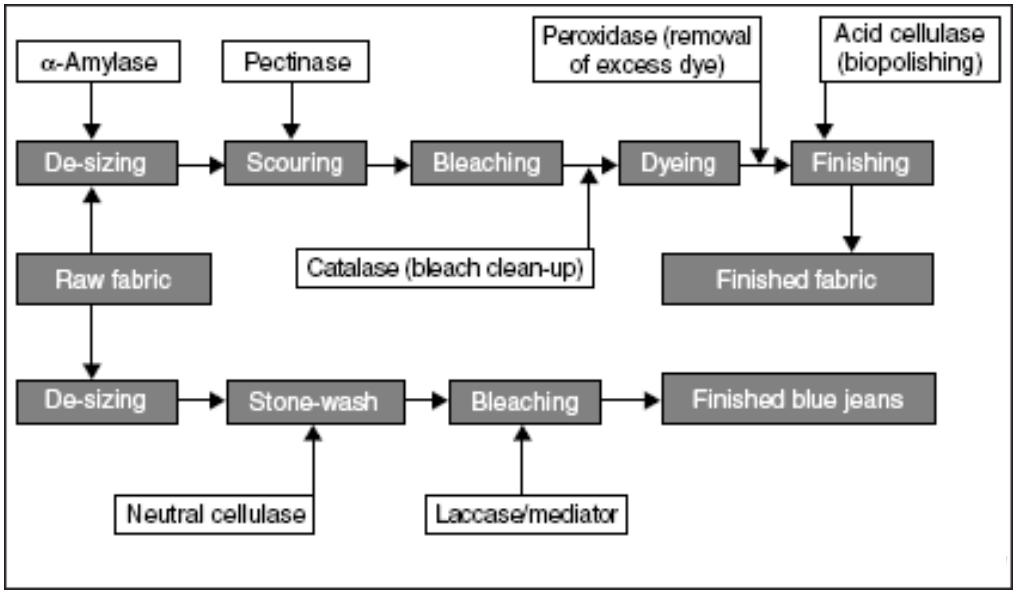

Fig. 1 Enzymes used in various unit operations in textile industry.

LACCASES

Origin and distribution

Laccase (benzenediol:oxygen oxidoreductase; p-diphenol oxidase EC 1.10.3.2), a blue copper containing oxidase, was first discovered by Yoshida (1883) in plants on the basis of the observation that the latex of the Chinese or Japanese lacquer trees (Rhus sp.) was rapidly hardened in the presence of air. The enzyme was named laccase about 10 years later after isolation and purification of the responsible catalyst (Bertrand, 1894). Other reports are those of Wosilait *et al.* (1954) on the presence of a laccase in leaves of *Aesculus parviflora* and in green shoots of tea (Gregory and Bendall, 1966). Five distinct laccases have been shown to be present in the xylem tissue of *Populus euramericana* (Ranocha *et al.*, 1999). Dicot plant laccase cDNA sequences have been reported from maple (*Acer pseudoplatanus*) (LaFayette *et al.,* 1995), tobacco (*Nicotiana tabacum*) (Kiefer-Meyer *et al.,* 1996), poplar (*Populus trichocarpa*) (Ranocha *et al.,* 1999), and yellow poplar (*Liriendron tulipifera*) (LaFayette *et al.,* 1999). In addition, the entire family of the *Anacardiaceae*, of which the lacquer tree is a member, appear to contain laccases in the resin ducts and in the secreted resin (Huttermann *et al.*, 2001). *Pinus taeda* tissue has been shown to contain eight laccases, all expressed predominantly in xylem tissue (Sato *et al.*, 2001). Monocot laccases from ryegrass (*Lolium perenne*) were recently cloned and characterized (Gavnholt *et al.,* 2002), and laccases have been found in all the major seed plant groupings. Furthermore, as laccase genes most likely were duplicated before and after division into monocots, dicots and gymnosperms, this further strengthens the contention that laccase genes are present in all higher plants, and probably in several copies (Gavnholt *et al.,* 2002). Laccases are usually present as several isoenzymes in lignifying tissue. Each laccase isoenzyme exhibits its own unique substrate specificity, complicating the study of these enzymes (Gavnholt *et al.*, 2002). Laccase cDNAs have also been isolated from cotton (*Gossypium arboreum*) (Dong Wang *et al.*, 2004).

Fungal laccases were also discovered during the 19th century (Bertrand, 1896; Laborde, 1896). Laccases are thought to be nearly ubiquitous among fungi, actually the presence of laccases has been documented in virtually every fungus examined for it. Laccases are produced in multiple isoforms depending on the fungal species and environmental conditions.

Some phenoloxidases showing “laccase properties” have been purified from larval and adult cuticles of insects such as *Drosophila virilis* (Yamazaki, 1969), the silk moth *Bombyx mori* (Yamazaki, 1972), *Lucilia cuprina* (Barrett, 1987a), *Sarcophaga bullata* (Barrett, 1987b), *Manduca sexta* (Thomas *et al.*, 1989), *Drosophila melanogaster* (Sugumaran *et al.*, 1992), and the malaria mosquito *Anopheles gambia*e (Dittmer *et al.*, 2004). Laccase genes in *M. sexta* and *A. gambiae* are expressed in multiple tissues in both a constitutive and regulated manner (Dittmer *et al.*, 2004).

Laccase activity was also found in the haemocytes of the red swamp crayfish (Cardenas and Dankert, 2000).

Until recently laccases were found only in eukaryotes. Now there are increasing evidences for the existence in prokaryotes of proteins with typical features of the multi-copper oxidase enzyme family (Alexandre and Zhulin, 2000; Claus, 2003).

Corresponding genes have been found in gram-negative and gram-positive bacteria, including species living in extreme habitats, e.g. in *Oceanobacillus iheyensis* or *Aquifex aeolicus* and in the archaebacterium *Pyrobaculum aerophilum*. Early reports of laccases in actinomycetes were based on rather non-specific substrate reactions, but have been verified for *Streptomyces griseus* (Freeman *et al.*, 1993; Endo *et al.*, 2002), and *Streptomyces lavendulae* (Suzuki *et al.*, 2003). The first convincing data for a prokaryotic laccase activity were presented for *Azospirillum lipoferum* (Givaudan *et al.*, 1993). A laccase-like enzyme activity was found in spores of a *Bacillus sphaericus* strain (Claus and Filip, 1997), and in the melanogenic marine bacterium *Marinomonas mediterranea* (Sanchez Amat and Solano, 1997). Recently, the spore protein CotA of *Bacillus subtilis* has been recognized to be a laccase (Hullo *et al.*, 2001).

Properties

More than 200 enzymes divided into oxygenases and oxidases, are known to use molecular oxygen (dioxygen) as one of their substrates (co-substrates). Only six of them are able to reduce dioxygen to two molecules of water. Besides cytochrome-*c* oxidase, a heme:Cu containing enzyme, only the blue oxidases (laccase, L-ascorbate oxidase, L-ascorbate:oxygen oxidoreductase; ascor-base), ceruloplasmin (Fe(II):oxygen oxidoreductase; ferrooxidase) are able to achieve this reaction. Bilirubin oxidase (bilirubin:oxygen oxidoreductase) and phenoxazinone synthase are classified into the group of so-called multicopper oxidases, too (Call and Mücke, 1997). All these 'blue', copper as an important cofactor containing enzymes differ remarkably in their primary amino acid sequence and their biological function, but they are rather similar in their three dimensional structure. The striking conservation at the active site level of very different copper-containing oxidases suggests that the enzyme activity linked to the copper sites must have been a very early biological event (Solomon *et al.* 1996). Therefore laccases are probably ancient enzymes from an evolutionary point of view (Messerschmidt and Huber 1990). Laccases have been extensively examined since the mid seventies and the results of these studies have been published in numerous articles which have been the subject of many reviews (Malmström *et al.*, 1975; Fee, 1975; Reinhammar, 1979, 1984; Thurston, 1994; Yaropolov *et al.*, 1994; Solomon *et al.*, 1996; Gianfreda *et al.*, 1999; Mayer and Staples, 2002; Claus, 2003). Among laccases, those of fungal origin are by far the most extensively studied.

Both plant and fungal laccases are glycosylated enzymes, the former ones showing a higher extent of glycosylation (22% to 45%) than the latter (10-25%). The range of substrates laccases can oxidize is very wide. Reports on molecular weight, pH optimum, substrate specificity and other properties of laccases show extreme diversity. To what extent this diversity is the result of isolation and purification procedures is unknown. Laccases have been isolated as monomeric, dimeric or even as tetrameric proteins in their active holoenzyme form, showing generally an acidic pI, and usually containing four copper atoms per monomer bound to three redox sites. Based on spectroscopic analysis, which reflects geometric and electronic features, copper centers are differentiated as type 1 (T1), or blue copper center, type 2 (T2) or normal copper, and type 3 (T3) or coupled binuclear copper centers (Solomon *et al.*, 1996). Type 1 copper has a trigonal coordination, with two histidines and a cysteine as conserved ligands. A methionine is considered to be a fourth axial ligand in ascorbate oxidase, whereas in

fungal laccases this site is occupied by a leucine or a phenylalanine. It has been postuled that the co-ordination geometry and ligands nature of the type-1 (T1) Cu might determine the redox potential (E°) of this metal. Actually, despite strong similarity in their EPR parameters, the reduction potential of the T1 center can vary widely in different enzymes, from approximately 0.465 V in *Myceliophthora thermophila* laccase to 0.775 V in *Polyporus versicolor* laccase. The redox potentials of the T1 sites in fungal laccases are generally much higher (≈ 0.3 V) than those of plant laccases and other blue copper oxidases (Xu *et al.*, 1996). The type 1 copper centers are characterized by unusually small hyperfine coupling constants for the paramagnetic the Cu(II) form of the proteins and an intense electronic absorption band nearly 600 nm (ε= 5000 cm^{-1}), which is responsible of their deep blue colour. This band is a cysteine sulphur to copper charge transfer transition (ligand to metal charge transfer, LMCT, band). The occurrence of laccase enzymes which lack the typical absorption around 600 nm has been reported. For example, a ‘‘white laccase’’ (containing 1Cu, 1Fe, 2Zn atoms) has been purified from *Pleurotus ostreatus* (Palmieri *et al.*, 1997), while ‘‘yellow laccases’’ (containing copper but in an altered oxidation state) have also been reported (Leontievsky *et al.*, 1997). Type 2 copper shows no absorption in the visible spectrum, and reveals paramagnetic properties in EPR studies. It is strategically positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm (oxidized form) and by the absence of an EPR signal as the result of the anti-ferromagnetic coupling of the copper pair. The type 3 copper centre is also the common feature of another protein superfamily including tyrosinases and haemocyanins (Decker and Terwilliger, 2000). Type 2 copper is coordinated by two and type 3 copper atoms by six histidines. The strong anti-ferromagnetical coupling between the type 3 copper atoms, is maintained by a hydroxyl bridge.

Multiple sequences alignment of more than 100 laccases resulted in identification of four ungapped sequence regions, L1-L4, as the overall signature of laccases, distinguishing them within the broader class of multi-copper oxidases (Kumar et al., 2003). The 12 amino acid residues in the enzymes serving as the copper ligands are housed within these conserved regions. The amino acid ligands of the trinuclear cluster, the eight histidines, occur in a highly conserved pattern of four HXH motifs. In one of these motifs, X is the cysteine bound to the T1 copper while each of the histidines is bound to one of the two type 3 coppers. Intra-protein homologies between signatures L1 and L3 and between L2 and L4 suggest the occurrence of duplication events. Laccases from basidiomycetes and ascomycete fungi have been crystallised, and their three-dimensional structure determined (Ducros *et al.*, 1998; Bertrand *et al.*, 2002; Piontek *et al.*, 2002; Hakulinen *et al.*, 2002). The global fold consists of three cupredoxin-like domains which are tightly associated resulting in a globular structure (Fig. 2). This molecular architecture of barrel domains is very similar to that found for other blue multi-copper enzymes, with the overall structure being most similar to that of a monomer of ascorbate oxidase. The T1 site (Cu1) belongs to domain 3 and sits at the base of a shallow depression. Type 1 copper is the site where substrate oxidation takes place, and the relatively large size of the groove, coupled with the huge sequence variability, presumably contribute to the broad substrate specificity displayed by fungal laccases.

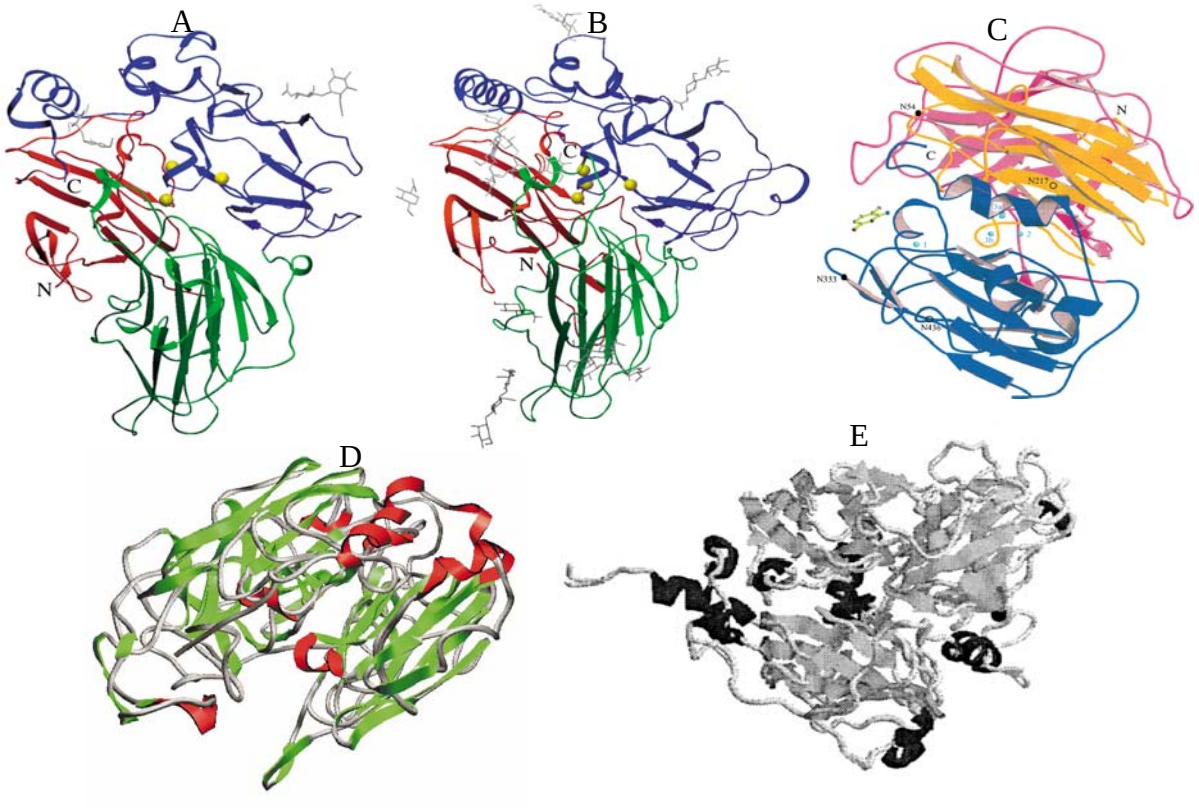

Fig. 2 Tertiary structures of laccases. A) *Coprinus cinereus* laccase crystal structure (Ducros *et al.*, 1998); B) *Melanocarpus albomyces* laccase crystal structure (Hakulinen *et al.*, 2002); C) *Trametes versicolor* laccase crystal structure (Bertrand *et al.*, 2002); D) model for a laccase homolog, PcoA, from *Escherichia coli* (Alexander and Zhulin, 2000); E) Predicted structures of ryegrass (*Lolium perenne*) laccase LpLAC5-6 (Gavnholt *et al.*, 2002).

The T2/T3 site is at the interface between the two other domains. Type 2 and type 3 copper form a trinuclear cluster, where the reduction of molecular oxygen and the release of water take place. The oxygen-reducing site at the T2/T3 clusters could have access to solvent through two channels, which lead to the type-3 copper sites and to the type-2 copper site, respectively. Solvent channels of the blue copper oxidases should be suited to allow fast access of dioxygen molecules to the trinuclear cluster and a subsequent easy release of water. In the ascomycete laccase structure (Fig. 2B) there is a narrower substrate-binding pocket. Moreover, the C-terminus on the *Melanocarpus albomyces* laccase forms a plug that blocks the access to the trinuclear-site tunnel. It has been reported a C-terminal processing for ascomycete laccases, and although the processed C-termini of these enzymes are not identical, they have a conserved Asp-Ser-Gly-(Leu/Val/Ile) sequence preceding the cleavage site. The reason for and the significance of C-terminal processing are not yet understood. The *M. albomyces* laccase structure suggests that the processed C-terminus may bind to the tunnel leading to the trinuclear copper site. The C-terminal processing and the conserved cleavage site also suggest that C-terminal blocking might be a general feature among ascomycete laccases. The tunnel could function as an access route for the oxygen molecule to enter the trinuclear copper site. The entrance of an oxygen molecule and the exit of a water

molecule through this tunnel would require a conformational change in the C-terminus of ascomycete laccase to open the tunnel. In any case, the closure of the tunnel certainly affects the function of the trinuclear copper site. Whether this is possible among basidiomycete laccases, which have different C-terminal residues, is not know. Interestingly, Gelo-Pujic *et al.* (1999) have noticed that the redox potential of a truncated basidiomycete laccase from *Trametes versicolor* (produced in *Pichia pastoris*) changed when 11 amino acids at the C-terminus were replaced with a single Cys residue. This suggests that the C-terminal amino acids might have a role in the function of all fungal laccases.

Basically any substrate with characteristics similar to a *p*-diphenol will be oxidized by laccases. In addition, the enzymes can also oxidize substrates ranging from inorganic/organic metal complexes, and ferrocyanide, anilines, benzenethiols, to other redox inorganic, organic or biological compounds, as long as their redox potentials are not too high (>1V). In general, laccases have low specificity for these reducing substrates, in contrast to their strong preference for O_2 as the oxidizing substrate.

The mechanism of electron transfer, as well as that of dioxygen reduction to water by blue-copper oxidases is an intriguing biochemical problem. Laccases catalyse the four one-electron reduction of dioxygen to water with four concomitant one-electron oxidations of a reducing substrate. A general reaction scheme has been proposed (Holm. *et al.*, 1996):

$4RH+O_2=4R+2H_20$.

The oxidation of a reducing substrate typically involves the formation of a free (cation) radical after the transfer of a single electron to laccase. The radical can further undergo laccase-catalysed oxidation (e.g., to form quinone from phenol) or non-enzymatic reactions (e.g., hydration or polymerisation) (Fig. 3).

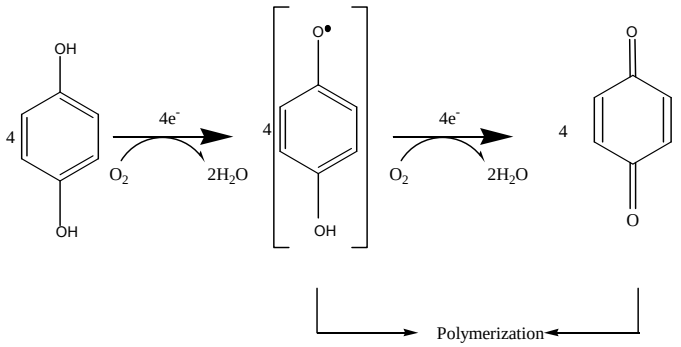

Fig. 3 A typical laccase catalysed reaction.

The T1 Cu site functions as the primary electron acceptor, extracting electrons from the reducing phenol substrate, and delivering them to the trinuclear site. The trinuclear center, the binding site for the second substrate, dioxygen, accepts electrons from the T1 site for reduction. The exact nature of the reaction scheme remains controversial with kinetics suggesting a “two-site ping-pong bi-bi” mechanism, which means that products are released before binding of new substrates occurs. The resting form of the enzyme is

likely to have all four copper oxidized as Cu (II) species with the T3 pair of Cu atoms bridged by a hydroxide ligand. The first step is the reduction of a single molecule of the reducing substrate by the T1 Cu. This electron is then transferred to the T2/T3 site, presumably through a conserved Cys-His pathway. Four one electron oxidations are required in order to fully reduce the enzyme; laccases has been therefore described as a molecular battery, accumulating electrons from individual oxidation reactions in order to reduce molecular oxygen. Reduction of oxygen most likely takes place in two, two-electron steps, since bound oxygen intermediates are involved. Transfer of two electrons generates a hydroperoxide intermediate which is reduced to two molecules of water (Fig. 4). The first is the rate-determining step, whereas the second step is supposed to be very fast. It is important to recognize that there is a large Franck-Condon barrier to reduction of peroxide associated with O-O bond fission. The large thermodynamic driving force associated with two-electron rather than the one-electron reduction is required to overcome this barrier for a high electron transfer rate, as it is observed in the native enzyme. Finally this form is reduced for further turnover or relaxes to the resting state.

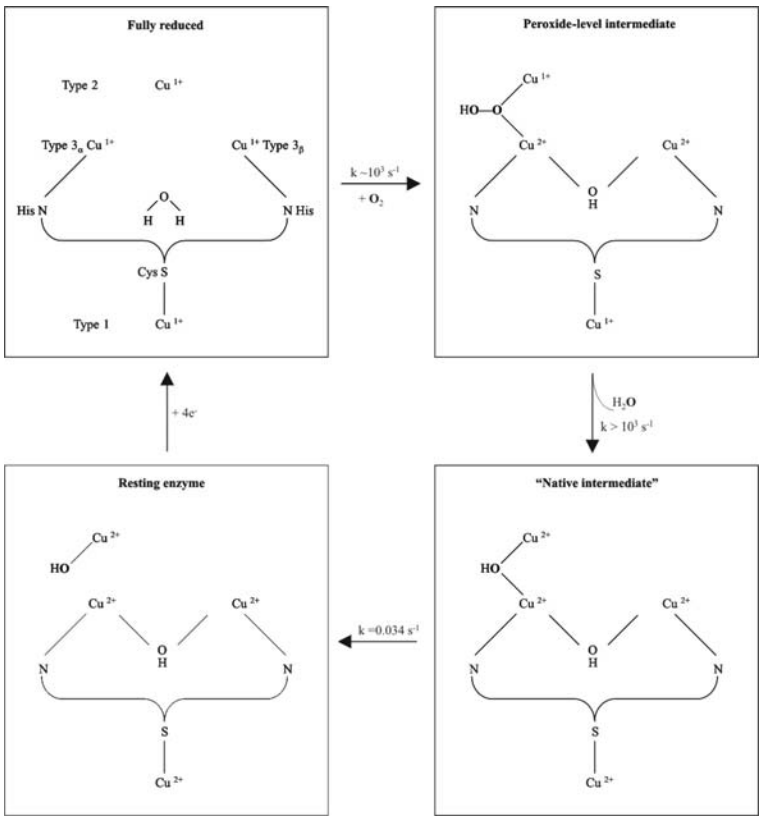

Fig. 4 Proposed reaction mechanism of dioxygen reduction in the multicopper oxidases (Shin *et al.*, 1996).

A comparative study of 5 enzymes and 40 substrates shows that the oxidation can be controlled by redox potential differences between the reducing substrate and the type 1 Cu (Xu *et al.*, 1996). A lower oxidation potential of substrate or a higher redox potential of laccases (type 1 site) often results in a higher rate for substrate oxidation, indicating

that the electron transfer (from the substrate to laccases) correspond to the Marcus' "outer-sphere" mechanism type. The substrate specificity and affinity of laccases can vary with changes in pH. For substrates whose oxidation does not involve proton exchange (such as ferrocyanide), laccase activity often decreases as pH increases, whereas for substrates whose oxidation involves proton exchange (such as phenol), the pH-activity profile of laccases can exhibit an optimal pH whose value depends on laccases rather than on substrate. For phenols, the optimal pH range is between 3 and 7 for fungal laccases and may increase to 9 for plant laccases (Gianfreda et al., 1999). This difference in pH-activity profile is attributed to the balance of two opposing effects: one generated by the redox potential difference between a reducing substrate and the type 1 Cu of laccases (which correlates to the electron transfer rate and is favoured for a phenolic substrate by higher pH), and the other generated by the binding of a hydroxide anion to the type2/type3 Cu of laccases (which inhibits the activity at higher pH).

Role in nature

Plants. Laccases were the first enzyme shown to be able to polymerise lignin monomers *in vitro* (Freudenberg *et al.*, 1958), and it was therefore concluded that these enzymes play the same role *in vivo*. One decade later, two papers (Nakamura, 1967; Harkin and Obst, 1973) were published that unambiguously concluded that laccases are not involved in lignin polymerisation. The hypothesis of laccase involvement in lignin polymerisation was therefore discredited. Recently, convincing evidences have shown that laccases are associated with the process of lignin polymerisation. Several studies indicated that laccases and laccase-like activities are closely correlated with lignin deposition in developing xylem (Davin *et al.* 1992, Dean and Eriksson 1994). A laccase isolated from *Acer* (maple) suspension cells (Bligny and Douce 1983) was not only immunolocalised in lignifying tissues of *Acer* (Driouch *et al.*, 1992) but was also capable of oxidizing and polymerising monolignols *in vitro* (Sterjiades *et al.*, 1992). In 1993, a laccase was purified for the first time from lignifying *Pinus taeda* xylem (Bao *et al.*, 1993). It has been suggested that since laccases operate in the absence of toxic H_2O_2, they could play a role in the early stages of lignification in living cells (Sterjiades *et al.*, 1993). This could, for example, be the case during the formation of lignified seed coats near sensitive developing embryos. It has also been suggested that laccases might be the principal lignification enzymes under conditions where lignin concentration has reached a level where the middle lamella has become so hydrophobic that most water and H_2O_2 is excluded, whereas O_2 is still available (Northcote, 1989). Eight Laccase cDNAs have been isolated in *Loblolly pine (Pinus taeda*) (Sato *et al.*, 2001): the fact that all of them are expressed predominantly in immature secondary xylem suggests that all of them play an important role in xylem formation, perhaps in lignin biosynthesis. Moreover, one of them (Lac 7) is expressed in every tissue and organ investigated except phloem. This may suggest that this isoenzyme has various functions besides lignin synthesis. One laccase from ryegrass stem of *L. perenne,* LpLAC5 4, does not contain an N-terminal signal peptide and is therefore unlikely to be secreted (Gavnholt *et al.*, 2002). Analysis of plant laccase sequences indeed suggests that a number of other laccases might also be localized intracellularly. Ferroxidase activity was detected in a laccase-like multicopper oxidase produced in transgenic tobacco cells expressing an LMCO cDNA (Lt*lacc2.2*) cloned from yellow-poplar (*Liriodendron tulipifera*). With respect

to ferroxidase activity in certain plant, a role in iron uptake has been proposed for laccases (Hoopes *et al.*, 2004).

Together, these data can be interpreted as a strong indication of laccases acting as a multifunctional enzyme in plants.

Fungi. Most, but not all, fungal laccases are extracellular, and a given species may produce isoenzymes of both extra- and intracellular types. One of the laccases produced by *P. ostreatus* shows activity inside the cell or in the cell wall (Palmieri *et al.*, 2000). Although differential expression of laccase isoenzymes has been documented for several basidiomycetes, there is no clear evidence of alternative function among them, as this has been documented for plant laccases. The great abundance laccases in wood-rotting basidiomicete fungi seems to indicate that the main role of fungal laccases is to depolymerise lignin, i. e., to break down into small components the highly polydispersed polyphenolic lignin macromolecule. However, this function contrasts with that of laccases in plant. As ascertained by studies conducted *in vitro* on small lignin model compounds (Hatakka, 1994; Youn *et al.*, 1995), the first step of laccase mediated lignin degradation is an oxidative reaction with the loss of one electron from phenolic hydroxyl groups of lignin to produce phenoxy radicals. The radicals may spontaneously reorganize and give rise to the cleavage of alkyl side chains of the polymer. Concurrently, due to the polymerising activity of the enzyme, the low-molecular-weight products may form polymeric compounds as well. These two contrasting actions suggest that lignin biodegradation by laccase-producing fungi probably occurs in nature through more complicated enzymatic mechanisms. The biodegradation process involves the synergistic effects of other enzyme (peroxydases, glyoxal oxidase, glucose oxidase, aryl alcohol oxidase) and non-enzymatic components that interact to establish an equilibrium between enzymatic polymerisation and lignin depolymerisation. In addition to their role in lignins transformation, fungal laccases seem to be involved in morphogenesis and/or growth of rhizomorphs in *Armillaria mellea* (Worrall *et al.*, 1986), conidia pigment production in *Aspergillus nidulans* (Clutterbuck, 1972), fruit body formation in *Lentinus edodes* (Leatham *et al.*, 1981), *Schizophyllum commune* (Leonard, 1971) and *Coprinus congregatus* (Ross, 1982), pathogenicity in several fungi such as *Cryptococcus neoformans* (Salas *et al.* 1996), *Cryphonectria parasitica* (Rigling *et al.*, 1991), and *Botrytis cinerea* (Viterbo *et al.*, 1994), as alternative oxidase in the respiratory pathway in *Podospora anserina* (Frese *et al.*, 1992), and in copper and iron homeostasis (Stoj *et al.*, 2003).

Insects. Evidence of laccase-like enzymatic activities in the cuticle of larval and adult insects led the author to associate this phenoloxidase activity with the sclerotizzation process in insects (Dittmer *et al.*, 2004).

Anthropods. Since laccases activity is important in cuticle sclerotisation, it is possible that in crayfish haemocytes this enzyme might be important in the formation of capsules around parasites too big to be phagocytosed (Cardenas and Dankert, 2000).

Bacteria. Role attributes to bacterial laccases include sporulation and pigmentation in *Bacillus sphaericus* (Claus and Filip, 1997) and *Marinomonas mediterranea* (Sanchez-Amat and Solano, 1997), and pigmentation of spores in *Bacillus subtilis* (Hullo *et al.*, 2001) conferring resistance to stress factors such as UV radiation or hydrogen peroxide (Martins *et al.* 2002).

Application of laccases

In addition to numerous studies on their strict biological functions, laccases are increasingly tested for their oxidative properties in a variety of practical applications ranging from hair dyeing process (Onuki *et al.*, 2000; Pruche *et al.*, 2000) to the generation of wood adhesives (Peshkova *et al.*, 2003).

Biopulping. About 25% of the wood pulp produced in the world is created using a mechanical pulping method, which has twice the yield of chemical pulping. Mechanical pulping has two disadvantages: it is energy intensive, and yields paper that is not as strong as paper produced from chemical processing. In many cases, chemical (Kraft) pulp is blended with mechanical pulp to add strength to the paper. However, chemical pulp is expensive and produces excessive amounts of air and water pollutants. Due to its importance in the pulp and paper industry, the removal of lignin from woody tissues is a process that has attracted a very great deal of research. Enzyme applications have been proposed for pulp and paper manufacture to enhance pulp bleaching, pulp refining, deinking, cellulose purification, deposit control, and papermaking (Wong and Mansfield, 1999). One of the approaches to delignification of wood fibres for preparation of pulp has been the use of laccases (with or without a mediator) for this purpose (biopulpuing). Biopulping results in nearly 30% saving of electricity, and biobleaching of pulp reduces chemical requirements of 50%. Moreover, laccases do not alter pulp brightness, and improve auto-adhesion of fibres in medium density fibreboard (Felby *et al.*, 1997), increase tensile strength of sheets derived of mechanical pulp (Buchert *et al.*, 1998), and preserve tensile strength through calendering (Wong and Mansfield, 1999).

Ethanol production. To improve the production of fuel ethanol from renewable raw materials, a laccase from the white rot fungus *T. versicolor* was expressed in *Saccharomyces cerevisiae* to increase its resistance to phenolic inhibitors present in lignocellulose hydrolysates. The laccase-producing transformant had the ability to convert coniferyl aldehyde at a very fast rate, which enabled faster growth and ethanol formation, showing a definite advantage of using laccase expressing yeast strains for producing ethanol from lignocellulose (Larsson *et al.*, 2001).

Treatment of beverages. Several phenolic compounds (cumaric acids, flavans, and anthocyanins) are usually present in beverages (wine, fruit juice and beer) and may, during their shelf life, cause undesirable and deleterious changes such as discolorouration, clouding, haze, and flavour changes. Positive effects of laccase action were observed on must and wine (Servili *et al.*, 2000) as well as on fruit juice.

Biosensors. The use of laccases as analytical tools is well documented by several reports (Bauer *et al.*, 1999; Freire *et al.*, 2003; Kulys *et al.*, 2003). Fungal laccases have been employed to estimate the phenolic content of natural juice (Cliffe *et al.*, 1994) or catechol in tea (Ghindilis *et al.*, 1992). The ability of laccases to catalyse the electroreduction of oxygen via a direct mechanism, without the presence of an electrochemically active mediator was used to develop a gas-phase oxygen biosensor, consisting of a laccase from *R. vernicifera*, with ascorbate as reducing substrate, both enclosed in pouches of low density polyethylene under nitrogen gas. In the presence of ascorbate, the blue chromophore prosthetic group of laccases was reduced and decolourised. When the enzyme was re-oxidised by oxygen, there was a concomitant return to the blue colour that is recorded both visually and spectrophotometrically at 610

nm. This oxygen biosensor is very active and stable. It was proposed as a useful tool to measure oxygen levels in products packaged under low oxygen concentrations whose quality and safety is strictly dependent on these low oxygen levels (Gardiol *et al.*, 1996).

Synthesis of antibiotics. Laccases have been employed to synthesize new cephalosporin antibiotics (Agematu *et al.*, 1993), and to improve the synthesis of actinocin antibiotics (Osiadacz *et al.*, 1999).

Bioremediation. Laccases have been reported to oxidize many recalcitrant substances, such as chlorophenols (Fahr *et al.*, 1999; Grey *et al.*, 1998; Ricotta *et al.*, 1996; Roy-Arcand and Archibald, 1991), lignin-related structures (Bourbonnais *et al.*, 1996; Boyle *et al.*, 1992), organophosphorous compounds (Amitai *et al.*, 1998), nonphenolic lignin model compounds (Kawai *et al.*, 1988; Majcherczyk *et al.*, 1999), phenols (Bollag *et al.*, 1988; Xu, 1996). Recent applications in bioremediation include an *ex planta* system of phytoremediaton of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase (Wang *et al.*, 2004). Laccases are also able to act on polycyclic aromatic hydrocarbons (PAHs) (Majcherczyk *et al.*, 1998), common, persistent and recalcitrant environmental contaminants with a tendency to bioaccumulate (Crawford and Crawford, 1996; Harvey, 1997). Many PAHs are toxic, carcinogenic and/or mutagens. They are generated by incomplete combustion of fossil fuels and other organic substances in petroleum refinery, manufactured gas production, wood treatment facilities and automobile exhaust as waste by-products (Durrant *et al.*, 1999; Sayler *et al.*, 1999).

Last but not least application in bioremediation is the treatment of industrial wastes, coming from olive oil mill and textile industries.

Olive oil production plays a leading role in the economy of most of Mediterranean countries. A number of by-products are produced during the olive oil production process. These are the pomace, which consists of mainly the pits of the fruits and solids after pressing, olive leaves, that are carried away with the olives as the latter are being collected, and a significant quantity of vegetable water of high organic load. The vegetable water (olive-mill wastewater, OMW) consists of the liquid fraction of the olive juice and the water used during the different phases of olive mill processing. OMW has significant polluting properties due to its high levels of chemical oxygen demand (COD), biochemical oxygen demand (BOD), and phenols. The direct impact that vegetable water has on the environment is the aesthetic degradation caused by its strong odour and dark colour. Furthermore, due to its high organic load, this vegetable water is likely to cause eutrophication in cases where it ends up in recipients where exchanging rates are low (closed gulfs, lakes etc). Of all components, polyphenols are the most problematic ones regarding their toxic properties, and their relatively slow degradability rate by specialized groups of microorganisms. On the other hand, polyphenols ensure that olive oil retains its quality over time (low acidity), acting as a natural preservative. Phenols contained in the OMW have a structure similar to lignin, which makes them difficult to biodegrade. In a recent study, the treatment of OMW with several laccase-producing fungi led to the removal of up to 78% of the initial phenolic compounds in 12/15 days (Tsioulpas *et al.*, 2002). This was associated to a decolourisation of the OMW from black to yellow-brown (Martirani *et al.*, 1996; Kissi et al, 2001) and to a

decrease of the phytotoxicity, as described by the Germination Index parameter (Fountoulakis *et al.*, 2002; Tsioulpas *et al.*, 2002).

Large amounts of structurally diverse dyestuffs are used for textile, leather, paper or food dyeing. Based on the chemical structure of the chromophoric group, dyes are classified as azo dyes, anthraquinone dyes, phthalocyanine dyes, etc. Out of these, azo dyes are commercially the most important group, making up about half of the total amount of dyestuffs used. The textile industry is by far the largest sector for dye utilization (800 kt per annum). All dyes used in the textile industry are designed to resist fading upon exposure to sweat, light, water, many chemicals including oxidizing agents, and microbial attack. During processing, up to 15% of the used dyestuff are released into the process water (Vaidya and Datye, 1982). Dye-containing effluents are hardly decolourised by conventional biological wastewater treatments (Shaul *et al.*, 1991; Willmott *et al.*, 1998). In addition to their visual effect and their adverse impact in terms of chemical oxygen demand, many synthetic dyes are toxic, mutagenic and carcinogenic (Michaels and Lewis, 1985; Chung *et al.*, 1992). Moreover, the frequently high volumetric rate of industrial effluent discharge, in combination with increasingly stringent legislation, makes the search for appropriate treatment technologies an important priority (O'Neill *et al.*, 1999). By far the class of microorganisms most efficient in breaking down synthetic dyes are the white-rot fungi. These constitute a diverse ecophysiological group comprising mostly basidiomycetes fungi capable of extensive aerobic lignin depolymerization and mineralisation. This is based on their capacity to produce one or more extracellular lignin-modifying enzymes, which, due to their lack of substrate specificity, are also capable of degrading a wide range of xenobiotics. Recently, *Trametes hirsuta*, and a laccase purified from the fungus, were used to degrade triarylmethane, indigoid, azo, and anthraquinonic dyes used in dyeing textiles (Abadulla *et al.*, 2000), as well as 23 industrial dyes (Rodriguez *et al.*, 1999). Immobilization of the *T. hirsuta* laccase on alumina enhanced the thermal stabilities of the enzyme and its tolerance against inhibitors such as halides, copper chelators, and dyeing additives. Treatment of the dyes with immobilized laccases reduced their toxicity up to 80% based on oxygen consumption rate of *Pseudomonas putida* (Abadulla *et al.*, 2000). Moreover, when the effluents were decolourised with immobilized laccase, they could be re-used for dyeing, and acceptable colour differences (ΔE^*) below 1.1 were measured for most dyes (Abadulla *et al.*, 2000).

Biotechnological and environmental applications require large amounts of enzymes. Laccases secreted from wild-type fungal organisms may not be suitable for commercial purposes, therefore, it is thought that their heterologous expression in different hosts could be a valuable approach to solve these problems.

RECOMBINANT EXPRESSION OF FUNGAL OXIDASES FOR INDUSTRIAL APPLICATION

The aim

Whether we consider fundamental studies to better understand the properties of the enzyme at the molecular and kinetic levels or their use for biotechnological and environmental applications, large amount of readily available crude and purified laccase are required. The practical use of laccase in applications has lead to search for new enzyme producers, the most suitable culture medium, the most appropriate, reproducible and inexpensive isolation procedures, and for mediators, which promote or facilitate enzyme action. Laccases secreted from wild-type plant or fungal organisms may not be suitable for commercial purposes mainly because low yields and undesirable preparation procedures (such as presence of toxic inducers) are not economically advantageous. Heterologous expression should be better suited for large-scale production, because of the potential of expressing different laccases in one selected optimised host. Laccases, like other oxidative enzymes, are difficult to express in non-fungal systems: table 2 summarizes recombinant laccases produced so far. It is worth to note the considerable variability of production yields with respect both to cDNAs expressed (originating from the same organism) and to the host used for the heterologous expression. As a fact, LCC1 laccase from *T. versicolor* was heterologously expressed in *P. pastoris* (Jönsson *et al.*, 1997) and not in *S. cerevisiae* (Cassland and Jönsson, 1999). Moreover, when *S. cerevisiae* was used as host, it was possible to note different level of expression between cDNAs originating from the same organism (Cassland and Jönsson, 1999; Klonowska *et al.*, 2004). Such “selectivity” in expression may reflect the inability of yeast to process different laccases post-translationally with the same efficiency (Klonowska *et al.*, 2004). Furthermore, these variable yields hinder forecast of the most suitable host, or the most promising laccase to express. Another current challenge for industrial applications is to improve the catalytic properties of natural laccases. For this purpose, the yeast *S. cerevisiae* has already been successfully used for directed evolution studies of a fungal laccase (Bulter *et al.*, 2003).

In order to get new insights into the structure/function/stability relationships of laccases, and with the aim to use directional evolution to create improved laccases with desirable physicochemical characters like a higher redox potential, a neutral or alkaline optimal pH, and a thermostability, this thesis was focused on the recombinant expression of laccases in yeast systems. Laccases from *Pleurotus ostreatus* were used as targets.

Laccases from *Pleurotus ostreatus*

The white-rot fungus *P. ostreatus* is able to express multiple laccase genes encoding isoenzymes with interesting properties considering both industrial applications structure-function studies. Amount of each specific enzyme produced depends on culture conditions. So far, five isoenzymes secreted by the mycelium have been purified and characterized: POXC (Palmieri *et al.*, 1993), POXA1w (Palmieri *et al.*, 1997), POXA1b (Giardina *et al.*, 1999), and, more recently, the two strictly related isoenzymes POXA3a and POXA3b (Palmieri *et al.*, 2003). POXC is the most abundantly produced in all

growth conditions tested so far; POXA1w shows peculiar differences with regard to metal ions content, in fact this enzyme contains two zinc atoms, one iron atom, and only one copper atom per molecule; POXA1b is the most stable at alkaline pH and shows activity inside the cell or on the cell wall (Palmieri *et al.*, 2000); POXA3a and POXA3b are heterodimeric laccases (Palmieri *et al.*, 2003). Studies on laccase encoding genes have also led to the identification of four different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*) (Giardina *et al.*, 1996), *pox1* (which codes for a laccase isoenzyme not identified yet) (Giardina *et al.*, 1995), *poxa1b* and *poxa3* (Giardina *et al.*, 1999; Palmieri *et al.*, 2003).

Table 2 List of heterologously expressed laccases.

Laccase	Source	Host	Comments	References
PO1	*C. hirsutus*	*S. cerevisiae*	Active laccase secreted in the medium.	Kojima *et al.*, 1990
PO2	*C. hirsutus*	*S. cerevisiae*	Active laccase secreted in the medium.	Kojima *et al.*, 1990
PrL	*P. radiata*	*T. reesei*	Laccase secreted activity of 7.7nkat ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Saloheimo *et al.*, 1991
LCC1	*R. solani*	*A. oryzae*	Laccase activity secreted in the medium. The enzyme was purified and partially characterized.	Wahleithmer *et al.*, 1996
LCC2	*R. solani*	*A. oryzae*	Active laccase secreted in the medium.	Wahleithmer *et al.*, 1996
LCC4	*R. solani*	*A. oryzae*	Laccase activity secreted in the medium. The enzyme was purified and partially characterized.	Wahleithmer *et al.*, 1996
LCC1	*T. villosa*	*A. oryzae*	Active laccase secreted in the medium. The enzyme was purified and partially characterized.	Yaver *et al.*, 1996
MtL	*M. thermophyla*	*A. oryzae*	Laccase secreted activity of 0.85 U ml^{-1} (SGZ). The enzyme was purified and partially characterized.	Berka *et al.*, 1997
LCC1	*T. versicolor*	*P. pastoris*	Active laccase secreted in the medium. Production yield was further optimised.	Jönsson *et al.*, 1997; O'Callaghan *et al.*, 2002; Hong *et al.*, 2002
LCC1	*T. versicolor*	*S. cerevisiae*	Undetectable laccase activity in the medium.	Cassland and Jönsson, 1999
LCC2	*T. versicolor*	*S. cerevisiae*	Active laccase secreted in the medium. Production of ethanol from raw materials.	Cassland and Jönsson, 1999 Larsson *et al.*, 2001
LAC	*S. commune*	*A. sojae*	Laccase secreted activity of 774 U ml^{-1} (Gallic acid).	Hatamoto *et al.*, 1999
LCCI	*T. versicolor*	*P. pastoris*	Active laccase secreted in the medium. The enzyme and a truncated version (LCCIa) were purified and partially characterized.	Gelo-Pujic *et al.*, 1999
LCC1	*C. cinereus*	*A. oryzae*	Transformants secreted from 8.0 to 135 mg of active laccase per liter. The enzyme was purified and partially characterized.	Yaver *et al.*, 1999
LtLACC2	*L. tulipifera*	*Tobacco cells*	Protoplasts retained laccase activity which could be measured once the protoplasts were lysed.	LaFayette *et al.*, 1999
LAC1	*P. cinnabarinus*	*P. pastoris*	Transformants secreted 8.0 mg l^{-1} of hyperglycosylated active laccase	Otterbein *et al.*, 2000
LAC2	*L. pine (P. taeda)*	*S. cerevisiae*	Yeast cells accumulated the expected fusion protein in insoluble fractions without degradation of products, but no laccase activity was detected.	Sato *et al.*, 2001
PPOA	*M. mediterranea*	*E. coli*	Production of recombinant protein, with the most of activity, located in the membrane fraction rather than in the soluble one.	Sanchez-Amat *et al.*, 2001
LCCIV	*T. versicolor*	*P. pastoris*	Laccase secreted activity of 0.15 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Brown *et al.*, 2002
LAC4	*P. sajor-caju*	*P. pastoris*	Transformants produced 4.85 mg l^{-1} of active laccase. The enzyme was purified and partially characterized.	Soden *et al.*, 2002
PPO	*S. tuberosum L.*	*L. esculentum L.*	Active laccases secreted in the medium conferring resistance to pathogen Pseudomonas syringae pv *tomato*.	Li and Steffens, 2002
LAC1	*P. cinnabarinus*	*A. niger*	77-fold increased activity (7000 U ml^{-1}) (ABTS) using A. *niger* signal peptide. The enzyme was purified and partially characterized.	Record *et al.*, 2002
LCCI	*T. versicolor*	*Z. mays L.*	Laccase activity was found in the seed, and variability in the amount was seen. The highest level was 0.55% TSP (respect to Total soluble protein)	Hood *et al.*, 2003
MtL	*M. thermophyla*	*S. cerevisiae*	Laccase secreted activity of 0.6 U l^{-1} (ABTS). Total activity was enhanced 170-fold by directed evolution.	Bulter *et al.*, 2003

LCS-1	*C. subvermispora*	*A. nidulans*	Laccase secreted activity of 0.23 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Larrondo *et al.*, 2003a
LCS-1	*C. subvermispora*	*A. niger*	Laccase secreted activity of 0.23 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Larrondo *et al.*, 2003a
MCO1	*P. chrysosporium*	*A. nidulans*	The recombinant characterised protein was not a typical laccase but rather a multi-copper oxidase with strong ferroxidase activity.	Larrondo *et al.*, 2003b
LCC	*F. lignosus*	*P. pastoris*	Laccase secreted activity of 5.95 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Liu *et al.*, 2003
STSL	*S. lavendulae REN-7*	*E. coli*	Production of almost 30 mg l^{-1}, with a very low activity, probably due to aggregation and precipitation.	Suzuki *et al.*, 2003
LAC1	*M. albomyces*	*S. cerevisiae*	200-fold increased production using α-factor signal peptide (2.8 nkat ml^{-1}) (ABTS).	Kiiskinen and Saloheimo, 2004
LAC1	*T. sp. strain C30*	*S. cerevisiae*	Activity barely detectable in the medium.	Klonowska *et al.*, 2004
LAC2	*T. sp. strain C30*	*S. cerevisiae*	Undetectable laccase activity in the medium.	Klonowska *et al.*, 2004
LAC3	*T. sp. strain C30*	*S. cerevisiae*	Laccase secreted activity of 0.5 U ml^{-1} (SGZ). The enzyme was purified and partially characterized.	Klonowska *et al.*, 2004
LAC1	*P. cinnabarinus*	*A. oryzae*	Laccase secreted activity of 0.84 U ml^{-1} (ABTS). The enzyme was purified and partially characterized.	Sigoillot *et al.*, 2004
LAC1	*G. arboreum*	*P. pastoris*	Active laccase secreted in the medium via both α-factor and native laccase signal peptide.	Wang *et al.*, 2004
LAC1	*G. arboreum*	*A. thaliana*	Active laccases secreted in the medium conferring resistance to phenolic compounds of plant origin.	Wang *et al.*, 2004
LACIIIb	*T. versicolor*	*Y. lipolytica*	Laccase secreted activity of 0.23 U ml^{-1} (ABTS). The enzyme was partly purified and partially characterized.	Jolivalt *et al.*, 2004
LAC1	*M. albomyces*	*T. reesei*	Laccase secreted activity of 230 mg l^{-1}. the highest production level was obtained in fed-batch culture (920 mg l^{-1}). The enzyme was purified and partially characterized.	Kiiskinen *et al.*, 2004

Heterologous expression

The extraordinary progress achieved in recombinant DNA technology over the last 25 years has opened up exciting areas of research related to the production of heterologous proteins. The development of high-yield expression systems is crucial to the production of sufficient quantities of recombinant proteins to allow their understanding and engineering.

The choice of a particular host strain for the expression of a heterologous gene is one of the most important factors for the feasibility of an expression system. This choice is based on the complexity of the protein to be expressed, the protein production levels, and the properties of host cell. *Escherichia coli* and *Bacillus* are the most commonly employed bacterial candidates. Considerable information is available on their genetics and physiological characteristics and they often offer very high yields in heterologous protein production. However, bacteria lack post-translational modification systems and often produce eukaryotic proteins that are misfolded, insoluble, or inactive. Yeasts offer the ease of microbial growth and gene manipulation found in bacteria along with the eukaryotic environment and ability to perform many eukaryote-specific post-translational modifications, such as proteolytic processing, disulfide bridge formation, and glycosylation. Yeasts are economical, usually give high yields, and are low demanding in terms of time and effort. However, for some proteins, yeasts are not suitable expression systems, due to their inability to perform certain complex post-translational modifications, such as prolyl hydroxylation and amidation, as well as some types of phosphorylation and glycosylation (Cregg and Higgins, 1995).

Starting from 1981, most of the recombinant proteins produced in yeasts have been expressed in *Saccharomyces cerevisiae* strains (Hitzeman *et al.*, 1981). The yeast *S. cerevisiae* is unique among eukaryotes in exhibiting fast growth both in the presence or absence of oxygen. *S. cerevisiae* has been used for thousand of years by mankind in brewing and baking and is regarded as GRAS organism (generally recognized as safe). An overwhelming wealth of information on genetics, molecular biology and physiology has been accumulated making this traditional species the best characterised eukaryotic system today. The sequencing of its entire genome has been completed (Goffeau *et al.*, 1996) and thousand genes are characterised. During the last decades, the availability of relevant structural genes and its use in genetic engineering have become a fascinating approach to create microbial strains which can produce valuable proteins in high amounts and quality. Because of the obvious advantage described above and of the various possibilities to influence the metabolic flux aiming to improve production yields, many heterologous proteins production processes are based on *S. cerevisiae*. Thus, it is not surprising that the first commercialised recombinant vaccine, the hepatitis B vaccine, is *S. cerevisiae* derived (Valenzuela *et al.*, 1982). Although most of the knowledge about genetics, physiology and cultivation techniques in yeast concerns *S. cerevisiae*, this model yeast has displayed several limitations as a host for heterologous proteins production, such as retention of the product within the cell or the periplasmic space and hyperglycosylation of secreted proteins (Buckholz and Gleeson, 1991).

A growing number of non-*Saccharomyces* yeasts have become accessible as expression systems for heterologous gene products. These alternative host organisms include *Hansenula polymorpha*, *Kluyveromyces lactis*, *Pichia pastoris*,

Schizosaccharomyces pombe, *Schwanniomyces occidentalis* and *Yarrowia lipolytica*. Among these, *K. lactis* has become a model system for studies on molecular physiology of so-called "Non-conventional Yeasts". Genetic studies of *K. lactis* began in the early 1960s. *Saccharomyces lactis* was the name of the yeast at that time. Since the pioneer work by Halvorson and collaborators on the genetics of "*Saccharomyces lactis*" (Herman and Halvorson, 1963a,b), several laboratories have developed genetically labelled strains. The recent interest in *K. lactis* comes from its ability to grow on cheap substrates, a weaker tendency to hypermannosilation of proteins than *S. cerevisiae*, a higher efficiency of secretion than *S. cerevisiae* in many cases (Gellissen and Hollenberg, 1997; Müller *et al.*, 1998), its Crabtree-negative status (Siso *et al.*, 1996), a low catabolite repression (Mulder *et al.*, 1995), its excellent fermentation characteristics (Fleer *et al.*, 1991a) and the existence of both episomal and integrative vectors (Wesolowski-Louvel *et al.*, 1996). Moreover, the regulation of primary carbon metabolism in *K. lactis* differs markedly from that in *S. cerevisiae* and reflects the dominance of respiration over fermentation, typical for the majority of yeasts. The absence of aerobic ethanol formation in this class of yeasts represents the major advantage for the "cell factory" concept, and heterologous proteins, even for human use, are currently produced on a large-scale basis in *K. lactis* cells. Examples of efficient production in *K. lactis* concern both proteins of therapeutic interest, such as interleukin-1β (Blondeau *et al.*, 1994a; Fleer *et al.*, 1991a), granulocyte colony-stimulating factor (Hua *et al.*, 1994), hepatitis B surface antigen (Martinez *et al.*, 1992) and human serum albumin (Blondeau *et al.*, 1994b; Fleer *et al.*, 1991b; Saliola *et al.*, 1999), and proteins with enzymatic activity, such as prochymosin (Van De Berg *et al.*, 1990), α-galactosidase (Bergkamp *et al.*, 1992) and glucoamylase (Bui *et al.*, 1996).

Despite the above cited advantages linked to expression in yeasts systems, an efficient expression of different genes is not a routine matter, as the unique structural features of each gene and of its transcribed mRNA preclude the adoption of a generally applicable expression method.

Several important factors that have an impact on expression must be considered, and among these, a key factor is the expression vector. A variety of vector systems for efficient expression of heterologous genes have been developed. Although the plasmids are designed to satisfy the special demands and requirements of the specific yeasts, they nevertheless follow similar construction principles or share to a large extent similar or even identical components. Most of them are shuttle vectors with a bacterial part (origin of replication and selection marker) serving the purpose of cloning and amplification in *E. coli*. The yeast portion contains element for the propagation in yeast, either 2-μm sequences or ARS element (Autonomous replicating Sequence) combined with a yeast CEN sequence (chromosomal centromere) for *S. cerevisiae,* and pKD1 (Chen *et al.*, 1986) for *K. lactis*. It is also possible to direct site-specific integration of a plasmid vector into the chromosome of the host strain. A range of different strong promoters is used in expression vectors; these promoter elements can either control a constitutive expression or can be induced by a specific component added to the culture medium. The tight regulation of inducible promoter is advantageous in cases where the expressed protein might interfere with cell growth. Correct termination of the heterologous transcript is provided by a terminator sequence, although reports exist on an efficient expression without such a sequence (Gellissen and Hollenberg, 1997). For

secretion and for appropriate modification of many proteins from higher eukaryotes, it is necessary to fuse the protein sequence to a N-terminal leader sequence that directs the nascent polypeptide into the secretory apparatus. The α -factor leader sequence from *S. cerevisiae* or the killer toxin leader sequence from *K. lactis* is the most used ones. In many cases- but not in all- the genuine leader sequence of the heterologous protein will also work.

Directed evolution

Since the beginning of large scale (recombinant) enzyme production for industrial applications, protein engineering methods have been applied to improve protein properties. The properties of proteins can be altered through site-directed mutagenesis or directed evolution (also called *in vitro*, experimental or laboratory evolution). The directed evolution approach is generally favoured for many industrial enzymes, owing to the difficulties of relating the desired application with the required properties, albeit a rational design approach based on structure-function relationship is widely used. In the late 1980s, protein molecules were altered by site directed or site-specific mutagenesis of their genes. This opened an era of protein engineering (Balland *et al.*, 1985; Garvey and Matthews, 1990; Wagner and Benkovic, 1990). Even though this provides a prudent and rational design or modification of protein structure, it is not suited to the generation of many novel biomolecules simultaneously in a single pot, and the limited knowledge of protein structure-function relationship and the approximate nature of computer-graphic modelling make it impracticable. To enhance the exploitation of biomolecular diversity, new strategies (e.g. directed evolution, error-prone polymerase chain reaction and gene shuffling) have been attempted for creating combinatorial libraries that have individual point mutations. Error-prone PCR employs a low fidelity replication step to introduce random point mutations at each round of amplification. This method has the advantage of being simple and easy to use, however, the power of this method is limited due to the small sizes of libraries that can be obtained. The technique of DNA shuffling came from mimicking natural recombination by allowing *in vitro* homologous recombination of DNA (Stemmer, 1994a,b). In this method, a population of related genes is randomly fragmented and subjected to denaturation and hybridisation, followed by the extension of 5’ overhang fragments by Taq DNA polymerase. The length of the fragment increases as a result of repeated PCR cycles. DNA recombination occurs when a fragment derived from one template primes a template with different sequences. In contrast to the error-prone PCR mutagenesis, DNA shuffling allows a more direct recombination of all beneficial mutations from any given round to generate multistep mutants with dramatically improved phenotypes. Currently, these two techniques, followed by screening and/or *ad hoc* developed selection procedures represent a valuable tool for engineering of enzymes (Arnold, 2001).

Laccases are good target for directed evolution approaches because knowledge of structure-function relations underlying their key properties is very limited. For most applications, laccase activity requires the presence of mediators. One example is electron transfer in biofuel cell applications. Direct electron transfer from the electrode is less efficient than transfer via a mediator (Yaropolov, 1994.). For the application of laccases in the cathodic compartment of a biofuel cell, the rate of direct electron transfer

is insufficient (Katz, *et al.*, 1999; Palmore, *et al.*, 1999). Mediators, however, are mostly toxic, instable, or expensive. Moreover, they lead to side products that can inactivate the enzyme (Li, *et al.*, 1999). To increase stability of laccases in the presence of mediators or to raise their activity without mediators would be a suitable goal for directed evolution. Activity of laccases towards many substrates is a function of the difference in electrochemical potential between enzyme and substrate (Xu, 1996.). Mutations that change the electrochemical potential would be of interest for understanding of intramolecular electron transfer in copper oxidases, and a higher oxidation potential would allow the conversion of a wide range of compounds that are poor substrates for natural laccases or are not converted at all.

The thesis

As an application of the power of recombinant DNA technology, this research was focused on recombinant expression of laccases from *P. ostreatus* in yeasts. The challenge was to design strategies to maximize the yield of recombinant proteins, in the attempts to raise production levels sufficient to enable deeper understanding of the structure/function relationships and, consequently, industrial enzyme applications.

Filamentous fungi are, at least so far, the best producers of recombinant laccases, with protein yields from 70 mg l^{-1} (Record *et al.*, 2002) to 230 mg l^{-1} (Kiiskinen *et al.*, 2004). However, considering the manipulations required producing laccases with desirable physicochemical characters (higher redox potential, more neutral optimal pH, and better thermostability), filamentous fungi are probably not the organisms of choice for expression. Moreover, yeasts also have the potential advantage, compared with filamentous fungi, to produce laccases free from any contamination by other enzymes involved in lignocellular biodegradation.

The yeasts of choice were *K. lactis*, a “non-conventional yeast”, and *S. cerevisiae* the conventional alternative, whose main characteristics and potentialities have been already described in the previous section. Moreover, since achieving functional expression is a good target for directed evolution, high recombination frequency added to the ease of manipulation made the budding yeast, *S. cerevisiae,* the most commonly used host organism in directed evolution (Morawski *et al.*, 2001). The laccase expression system developed in this host was used to realize preliminary researches in tailoring enzymatic function.

1 EXPERIMENTAL

1.1 *Microorganism manipulation*

1.1.1 Bacteria

The *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.

1.1.1.1 Growth conditions

The medium used for growing *E. coli* was the rich medium Luria-Bertani. Bacterial cultures were conducted at 37°C. Bacterial cells competent to chemical transformation were obtained from cells grown on SOB medium.

LB	SOB
Bacto tryptone (Difco) 1%	Bacto tryptone (Difco) 2%
Yeast extract (Difco) 0.5%	Yeast extract (Difco) 0.5%
NaCl 1%	NaCl 10 mM
	$MgSO_4$ 20 mM
	$MgCl_2$ 10 mM

Selective medium was supplemented with 100 µg ml^{-1} of ampicillin; solid medium contained 15% of agar (Difco).

1.1.1.2 Competent cells preparation

Cells were picked up from a solid culture and grown in 5 ml of SOB medium at 37° C for 16 hours on a rotary shaker (250 rpm). The culture was then diluted 1:100 in the same medium and grown up to an optical density at 600 nm of 0.6-0.7. Growth is stopped by putting the culture on ice for 20 min., and cells are sedimented by centrifugation at 3000 x g at 4°C for 10 min. Cells were washed twice by incubation with cold $CaCl_2$ 0.1 M for 20 min on ice. Cell pellet was then resuspendend in $CaCl_2$ 0.1 M, and Glycerol 15% was added to the suspension. After vigorous mixing, the suspension was aliquoted and promptly frozen in dry-ice/acetone bath.

1.1.1.3 Competent cells transformation

Competent cells was thawed and incubated with transforming DNA plasmid (up to 100 ng) for 30 min. on ice. Mixture was then shocked by incubation at 42°C for 90 sec. and on ice for the same time. Cells were spread on selective solid LB medium after 1 hour incubation at 37 °C in LB medium.

1.1.1.4 Mini-preparation of plasmid DNA (alkaline lysis method)

Bacterial cells grown on solid LB medium were inoculated at 37°C over night in 3 ml of selective LB on a rotary shaker (250 r. p. m.). 1.5 ml of culture was centrifuged for 30 sec. at 13000 x g at room temperature. The cellular pellet was resuspended in 100 µl of cold GTE (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8). 200 µl of 0.2 N NaOH, 1% SDS were added to the cellular suspension. After a vigorous shaking, the solution was incubated on ice for 5 min to denaturate the cellular proteins. Then 150 µl of K-acetate 5M pH 5.2, were added, and the solution was incubated on ice for 5 min to allow the precipitation of genomic DNA. The suspension was centrifuged for 5 min at 13000 x

g at room temperature. The supernatant, containing plasmidic DNA, was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The plasmidic DNA was then precipitated by adding 2.5 volume of 100% EtOH at room temperature and rinsed with cold 70% EtOH. The plasmidic DNA was resuspended in 50 µl of TE pH 8 (10 mM Tris HCl pH 8, 1 mM EDTA), RNAasi A (150 µg ml^{-1}) and incubated at 37°C for 30 min.

1.1.2 The yeast *Kluyveromyces lactis*

The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (**a** *thr lys pgi1 adh3 adh1::URA3 adh2::URA3*).

1.1.2.1 Growth conditions

K. lactis was grown in YPPD medium at 28°C. Cultures were conducted at 28°C. Selective medium was YPPD supplemented with 100 µg ml^{-1} of geneticin, solid medium contained 15% of agar (Difco). Composition and concentration of selective medium was changed to try to improve the recombinant enzyme production. Plate assay was performed on solid selective medium supplemented with enzyme substrate: 0.2 mM ABTS (2,2'–azinobis (3ethylbenzothiazoline-6-sulfonate)) or 0.1 mM DMP (2,6–dimethoxy-phenol). Several inducer (EtOH) and cofactor concentrations (copper as $CuSO_4$) were tested in order to increase laccase production.

Component	YPPD	YPD	YPPG	YPPDG
Yeast extract (Difco)	1%	1%	1%	1%
Bacto tryptone (Difco)	4%	2%	4%	4%
Glucose	2%	2%		2%
Galactose			2%	2%

1.1.2.2 Yeast transformation

Cells were picked up from a solid culture and grown in 50 ml of YPPD medium at 28° on a rotary shaker (150 rpm) up to 1.3-1.5 OD_{600}. Cells were sedimented by centrifugation at 3000 x g at 4°C for 10 min and washed twice with cold H_2O, and once with cold 1 M sorbitol. Cell pellet was then resuspendend in 0.3 ml of 1 M sorbitol, and an aliquot of this suspension (50 µl) was mixed with transforming DNA plasmid (up to 1 µg). *K. lactis* transformation was performed by electroporation with a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. The cells were then spread on YPPD medium containing 100 µg ml^{-1} of geneticin G418, after an over night incubation at 28°C in YPPD. Agar plate assays on YPPD supplemented with 100 µg ml^{-1} of geneticin G418, 2% ethanol, 0.1 mM $CuSO_4$ and 0.2 mM ABTS (2,2'–azinobis (3ethylbenzothiazoline-6-sulfonate)) were used for transformant selection. Plates were incubated inverted for 4 days at 28°C and checked for the development of green colour. 100 µl of ethanol was added each day to the lid of the plate to compensate its evaporation.

1.1.2.3 Yeast cultivation and laccase production

30 transformed clones for each expression vector were screened for laccase production by growing them in 10 ml selective medium (YPPD containing geneticin) supplemented with 0.1 mM $CuSO_4$ and 2% ethanol, at 28°C on a rotary shaker (150 r.p.m.). 2% ethanol was daily added and samples were taken at intervals for optical density and laccase activity determination. The best producing clones were chosen to study laccase production. Inocula grown on selective YPPD medium were used to seed flasks (250 ml)

containing 50 ml of selective medium, starting from 0.06 OD_{600}. Cultures were grown at 28°C on a rotary shaker for 4 days. Preparative culture was performed in 1l flasks containing 200 ml of medium.

1.1.3 The yeast *Saccharomyces cerevisiae*

The *Saccharomyces cerevisiae* strain used for heterologous expression was W303-1A (*MAT ade2-1*, *his3-11*, *15*, *leu2-3*, *112*, *trp1-1*, *ura3-1*, *can1-100*).

1.1.3.1 Growth conditions

The medium used for growing wild-type *S. cerevisiae* was the rich medium YPD (1% Yeast extract (Difco), 2% Bacto tryptone (Difco), 2% Glucose). Cultures were conducted at 28°C. Solid medium contained 15% of agar (Difco). Transformed yeast was grown in a selective medium. Composition and concentration of selective medium was changed to try to improve the recombinant enzyme production.

Component	SD	Sd	SG	Sg
Yeast nitrogen base w/o AA (Difco)	0.67%	0.67%	0.67%	0.67%
Casaminoacids (Difco)	0.5%	0.5%	0.5%	0.5%
Glucose	2%	1%		
Galactose			2%	1%
Adenine	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}	30 mg l^{-1}
Tryptophane	40 mg l^{-1}	40 mg l^{-1}	40 mg l^{-1}	40 mg l^{-1}
Succinate buffer pH 5.3	50 mM	50 mM	50 mM	50 mM

Plate assay was performed on solid selective medium supplemented with enzyme substrate: 0.2 mM ABTS or 0.1 mM DMP (2,6–dimethoxy-phenol).

1.1.3.2 Yeast transformation

Yeast cells were scraped from an YPD plate and washed with 1 ml of H_2O. The cell pellet was then incubated in 1 ml of 0.1 M LiAc for 5 min at 30° C. Cells were sedimented by centrifuging at 12000 x g for 5 sec, resuspended in a mixture containing

- 240 µl of PEG (50% w/v)
- 36 µl LiAc 1 M
- 25 µl SS-DNA (2.0 mg ml^{-1})
- transforming DNA plasmid (up to 5 µg)
- 45 µl of H_2O

and incubated at 42° C for 20 min. Cells were then centrifuged, resuspended in H_2O, and spread on selective medium: plates were incubated inverted for 4 days at 28°C and checked for the development of green or orange colour.

1.1.3.2.1 Homologous recombination

Experiments of homologous recombination were performed following the previous protocol and transforming the yeast cells with different ratios (1: 1 and 1:3) of linearized plasmid (1.5 µg) and mutated cDNA.

1.1.3.3 Yeast cultivation and laccase production

Precultures (10 ml) were grown on selective SD medium at 28° C on a rotary shaker (150 rpm). A volume of suspension sufficient to reach a final OD_{600} value of 0.5 was then used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of selective medium and cells were then grown on a rotary shaker. Culture aliquots (1 ml) were daily collected,

centrifuged at 12000 g for 2 min at 4°C and assayed for optical density and laccase activity determination. Preparative cultures were performed in 1L flasks containing 200 ml of medium.

1.1.3.4 Mini-preparation of plasmid DNA (Robzyk and Kassir, 1992)

Yeast cells were grown in 3 ml of liquid SD medium at 28°C over night on a rotary shaker (150 r. p. m.). 1.5 ml of culture was harvested for 5 min. at 5000 x g at room temperature, and the pellet was re-suspended in 100 µl of cold STET (8% sucrose, 50 mM Tris-HCl pH 8, 50 mM EDTA, 5% Triton X-100). Cells were lysated by adding 0.2 g of 0.45 mm glass beads and vigorously vortexing. Following steps were: addition of another 100 µl of STET, briefly vortexing and incubation in a boiling water bath for 3 min. The suspension was then cooled on ice and centrifuged for 10 min. at 5000 x g at 4°C. To 100 µl of the supernatant was added 2.5 M ammonium acetate: the sample was incubated at –20°C for 1 h. and centrifuged for 10 min. at 12000 x g at 4°C. At this stage, residual chromosome DNA, large RNA species, and the putative impurities which inhibit *E. coli* transformation are precipitated. To 100 µl of the supernatant were added 200 µl of ice-cold ethanol, and DNA was recovered by centrifugation. The pellet was washed with 70% ethanol and re-suspended in 20 µl of water. 10 µl of this solution were used to transform competent bacteria.

1.2 *Nucleic acid manipulation*

All DNA manipulations were performed according to Sambrook *et al.*, (1989).

1.2.1 Cloning vectors

Plasmids used for cloning procedures were pUC13 (Amersham Biosciences), pUC18 (Amersham Biosciences) and pGEM7Zf (-) (Promega).

1.2.2 Expression vectors

Generally, these plasmid vectors ("shuttle vectors") contain genetic material derived from the *E. coli* vector pBR322 (or its derivatives) and a genetic element (origin of replication), which enable them to be propagated in *E. coli* cells prior to transformation into yeast cells and a selectable marker (mainly the β-lactamase gene, *bla*) for the bacterial host. Additionally, the shuttle vectors enharbor a selectable marker to be used in the yeast system. Conventionally, markers are genes encoding enzymes for the synthesis of a particular amino acid or nucleotide, so that cells carrying the corresponding genomic deletion (or mutation) are complemented for auxotrophy or autotrophy. Besides auxotrophic markers, G418 resistance can also be used (aminoglycoside phosphotransferase gene, *aph*). G418 is a ribosomal inhibitor in many eukaryotic cells. Principally, four types of shuttle vectors can be distinguished by the absence or presence of additional genetic elements:

Integrative plasmids (**YIp**) which by homologous recombination are integrated into the host genome at the locus of the marker, when this is opened by restriction and linearized DNA is used for transformation. This (normally) results in the presence of one copy of the foreign DNA inserted at this particular site.

Episomal plasmids (**YEp**) which carry part of the 2 μ plasmid DNA sequence (or pKD1 replication origin) necessary for autonomous replication. Multiple copies of the transformed plasmid are propagated in the yeast cell and maintained as episomes.

Autonomously replicating plasmids (**YRp**) which carry a yeast origin of replication (ARS sequence) that allows the transformed plasmids to be propagated several hundred-fold.

Cen plasmids (**YCp**). In addition to an ARS sequence these vectors carry a centromeric sequence (derived from one of the nuclear chromosomes) which normally guarantees stable mitotic segregation and reduces the copy number of self-replicated plasmid to just one.

Yeast expression vectors will employ promoter and terminator sequences in addition to the gene of interest. It is advantageous to use yeast-derived (homologous) rather than heterologous sequences, because the former are more efficient, and heterologous elements will sometimes not work in yeast. Constitutive promoters are derived from genes of the glycolytic pathway, because these lead to high-level transcriptional expression. On the other hand, regulated promoters can be controlled by controlling the availability of certain nutrients. This allows augmenting yeast cell mass prior to heterologous gene expression, so that the cell population can be optimised before the regulated promoters are turned on.

Protein secretion in yeast is a complex process and there is no generally accepted signal sequence which directs secretion. Although several foreign proteins can be

secreted under the direction of their own signals, homologous signal sequences are often more successful and can result in highly expressed heterologous proteins recoverable from the extracellular medium. Frequently used signal sequences in *S. cerevisiae* include those derived from invertase (*SUC2*), acid phosphatase (*PHO5*) or a-factor pheromone (*MFa1*). Secretion of foreign protein in *K. lactis* can be directed mainly, but not only, by the killer toxin signal peptide.

1.2.3 Polymerase chain reaction (PCR)

The expression vectors were constructed with a PCR cloning approach, and the cloned PCR products were checked by sequencing. Table 1.1 shows all the primers used in the PCR experiments and the corresponding annealing temperatures.

Table 1.1 List of the primers used in amplification experiments. Nucleotides in bold are complementary to the laccase cDNA sequences. Nucleotides in italics are recognized by restriction enzymes.

Primer	Nucleotide sequences	Ann. T
Fwpoxc	TTT*GAATTCAAGCTT***ATGTTTCCAGGCGCACGG**	60°C
Revpoxc	**GGCCACGATAT*GGTACC*AATCC**	60°C
Fwpoxa1b	ATAA*GAATTCAAGCTT***ATGGCGGTTGCATTCG**	54°C
Revpoxa1b	**GTGTGCCCGACA*AGATCT*GCAACAAC**	54°C
A1bfw	AAAA*GAATTCATGCAT*CT**AGCATTGGGCCCCGCGG**	54°C
A1brev	TAA*GGATCCGAATTC***TTATAATCATGCTTTCAATGGC**	54°C
FwBglII	**TGTTGC*AGATCT*TGTCGG**	54°C
Cfw	AAAA*GAATTCATGCAT*CT**GCCATTGGGCCCGCTGGC**	60°C
FwEcorV	**AGCC*GATATC*AACATCAATCTCG**	60°C
Crev	AAAA*TCTAGAGAATTC***TTAAGAAGTAGGAATAAGAAG**	60°C

The reaction conditions were optimised using the DNA polymerase from *Thermus aquaticus* (*Taq*) (Promega) The reaction mix (50 µl) contained cDNA as template (50 ng), primers (50 pmol), Taq buffer 1x, $MgCl_2$ 2mM, dNTP 200µM and milliQ water. 2.5 U of Taq polymerase were added after an extensive denaturation (hot start PCR). The polymerisation (72°C) was performed in a thermal cycler PCR-sprint Hybaid (Hybaid Ltd., Ashford, UK). Fig. 1.1 shows the cycle performed for the reaction, where X s the annealing temperature.

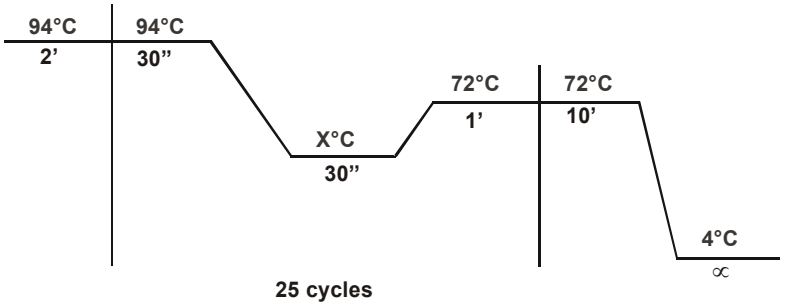

Fig. 1.1 Scheme of PCR reaction.

The annealing temperature depends on the primers used, whilst the time of the amplification depends on the fragments length (1min/1Kb). It has to be noted that the Pfu polymerase works at 74°C.

Once optimised the reaction conditions, polymerisation was performed using *Pfu* polymerase (Stratagene): the reaction mix (50 µl) contained cDNA as template (50 ng), primers (25 pmol), Pfu buffer 1x, dNTP 200µM and milliQ water. 2.5 U of Pfu polymerase (polymerisation temperature 74°C) were added after 2 min of extensive denaturation (hot start PCR) and the reaction followed the scheme reported in Fig. 1.1.

1.2.3.1 Random prone PCR

Mutazyme® DNA polymerase (GeneMorph PCR mutagenesis kit, Stratagene) is the enzyme used for random prone PCR.

Mutazyme randomly introduces errors more frequently than *Taq* DNA polymerase, and it produces a unique mutational spectrum.

The desired mutation frequency can be achieved selecting the amount of cDNA used as template, as higher mutation frequencies are reached simply by lowering input DNA template concentration and, consequently, increasing the number of replication cycles. The amount of cDNA used is:

- 10-100 ng $\Rightarrow$ 0-3 mutations/kb (low range);
- 10pg-10 ng $\Rightarrow$ 3-7 mutations/kb (medium range);
- double or triple PCR $\Rightarrow$ 7-16 mutations/kb (high range).

The reaction mix (50 µl) contained cDNA template, primers (250 ng μl^{-1} each), Mutazyme buffer 1x, dNTP 200µM and milliQ water. 2.5 U of Pfu polymerase (polymerisation temperature 72°C) were added after 2 min of extensive denaturation (hot start PCR). The reaction followed then the scheme in Fig. 2.1.

1.2.4 DNA modifying reactions

1.2.4.1 Digestion with restriction enzymes

The PCR products and the cloning vectors were hydrolysed with opportune restriction enzymes (Promega). The reaction was performed using 5U of enzyme each µg of DNA incubating at 37°C for 2 h and 30 min.

1.2.4.2 Dephosphorylation of the linearised plasmid

Calf intestinal alkaline phosphatase (CIP) (Boehringer Mannheim) is the enzyme of choice to remove 5'-phosphate groups from linear plamids to avoid a background

reaction of closed empty vector. The reaction was performed using 1 U of enzyme with 2 pmol of 5'-phosphate termini. The reaction mix (100 µl) was incubated a 37°C per 15 min., then at 55°C for 45 min., finally the enzyme was inactivated with 1 mM EDTA at 75°C for 10 min.

1.2.4.3 Ligation of foreign cDNA in a plasmid

The ligation was performed with ligase from T4 bacteriophage (Boehringer Mannheim) at 16°C over night. A maximum of 100 ng of plasmid was used for each reaction, and several molecular ratio plasmid: insert were tested (1:2, 1:3, 1:4), in a mix containing 1mM ATP, ligase buffer 1x and 1 U of enzyme.

1.2.5 Gel electrophoresis of DNA

The PCR products were separated and analysed on agarose gel 1% w/v. The electrophoresis was conducted in TAE (40 mM tris-acetate, 1mM EDTA pH 8) at 100 V. To visualize DNA 0.5 µg ml^{-1} ethidium bromide was added to the agarose gel. This substance contains a planar group that intercalates between the stacked bases of DNA. DNA fragments were visualized with ultraviolet light (365 nm).

PCR products and linear plasmid were extracted and purified from agarose gel with the kit Quiaex from Quiagen as specified by the manufacturer.

1.3 *Protein manipulation*

1.3.1 Assay of laccase activity

Culture aliquots (1 ml) were daily collected and cells were removed by centrifugation (12000 g for 2 min 4°C). Laccase activity in the culture supernatant was assayed at 25°C, monitoring the oxidation of ABTS at 420 nm (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}): the assay mixture contained 2 mM ABTS, 0.1 M Na-citrate buffer, pH 3.0.

Laccase activity towards DMP was assayed in a mixture containing 1 mM DMP and the McIlvaine's citrate-phosphate buffer adjusted to pH 5.3. Oxidation of DMP was followed by an absorbance increase at 477 nm (ε_{477}= 1.48 x 10^4 M^{-1} cm^{-1}).

Kinetic parameters (k_{cat} and K_M) were estimated using the software Micromath Scientist for Windows on a wide range of substrate concentrations.

Phenol oxidase stability at pH was measured using a McIlvaine's citrate-phosphate buffer adjusted to different pH level in the range 2.5-8.0. Stability at pH 10 was measured in 50 mM Diethanolamine-HCl buffer.

Phenol oxidase stability at 60°C was measured in 50 mM NaP pH 7.0.

1.3.2 Electrophoresis and isoelectric focusing (IEF)

Polyacrylamide (9%) gel slab electrophoresis in 0.1% SDS was carried out as described by Laemmli (1970). Cell lysates were obtained resuspending cellular pellets in the SDS loading buffer and incubating them for fifteen minutes at 90°C, vortexing each five minutes to help the cell disruption. Proteins were visualized by Comassie or Silver staining.

Analytical IEF in the pH range 1.5-9.5 was performed on 4.6% acrylamide gel slab with a Multiphor electrophoresis system (Amersham Biosciences), according to the manufacturer's instructions.

Native PAGE was performed at alkaline pH under non-denaturing conditions. The separating and the stacking gels contained 9% and 4% acrylamide, respectively: the buffer solution used for the separating gel contained 50 mM Tris-HCl (pH 9.5), and 18 mM Tris-HCl (pH 7.5) for the stacking gel. The electrode reservoir solution was 25 mM Tris-HCl and 190 mM glycine (pH 8.4). Gels were stained to visualize laccase activity by using ABTS as the substrate.

1.3.3 Western blotting

250 µl of yeast culture supernatants (10-fold concentrated), or 500 µl of *S. cerevisiae* B-pSAL4 culture supernatant (20-fold concentrated), and an amount of cellular lysate corresponding to 0.7 OD_{600}, were loaded on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Immobilion P, Millipore). Electroblotting was performed in 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid), pH 11.0, 10% (v/v) methanol at 50 V for 180 min at room temperature. Washing solution was 5% (w/v) dried milk, in phosphate-buffered saline solution supplemented with 0.2% (v/v) Triton X100 (washing buffer). The membrane was washed and incubated with rabbit anti-POXC antibodies diluted 1:5000 or rabbit anti-POXA1b antibodies diluted 1:2000 in washing buffer at room temperature for 1 h, under continuous shaking. Subsequently, the membrane was washed and incubated as above described with anti-rabbit IgG,

peroxidase conjugate (Sigma), diluted 1:2000 in washing buffer. Blots were visualised with 100 mM Tris-HCl, pH 7.5, 0.5 mg ml^{-1} 3,3'-diaminobenzidine, 0.03% (w/v) $NiCl_2$, 0.006% (v/v) H_2O_2.

1.3.4 Laccase purification

1.3.4.1 Recombinant laccases in *K. lactis*

Culture media were harvested on the optimal laccase production day, cells were sedimented by centrifugation at 12000 g at 4°C for 15 min. 1 mM PMSF was added to culture supernatant, and secreted proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4°C and centrifuged at 10000 g for 40 min. After this step, different purification strategies were exploited for rPOXA1b and rPOXC.

The rPOXA1b ammonium sulphate precipitate was resuspended in 50 mM Na-phosphate pH 6.0, and, after extensive dialysis against the same buffer, loaded onto a SP Sepharose Fast Flow (Amersham Biosciences) column equilibrated with the phosphate buffer. After washing, the enzyme was eluted at a flow rate of 1 ml min^{-1} with a linear gradient from 0 to 0.4 M NaCl. The active fractions were pooled, concentrated and desalted on an Amicon PM-30 membrane.

The rPOXC ammonium sulphate precipitate was resuspended in 50 mM Na-phosphate pH 7.0 containing 1 M $(NH_4)_2SO_4$, and loaded onto a Phenyl Sepharose High Performance 35/100 column (Amersham Biosciences) equilibrated with the same buffer. After an isocratic step at 1 M $(NH_4)_2SO_4$, a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ was applied. The active fractions were pooled, concentrated and desalted on an Amicon PM-30 membrane. Then, the sample was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) column equilibrated with the phosphate buffer and eluted as described above for rPOXA1b from ionic exchange chromatography. The active fractions were pooled, concentrated and desalted as above.

1.3.4.2 Recombinant laccases in *S. cerevisiae*

rPOXA1b. Culture media were harvested on the optimal laccase production day, cells were sedimented by centrifugation at 1600 g at 4°C for 15 min. 1 mM PMSF was added to culture supernatant, and secreted proteins were filtered throw whatmann sheet. The sample was concentrated with a Quixstand Benchtop system (Amersham Biosciences) throw a cartridge with cut-off 30000 NMWLC, and extensively dialysed against 50 mM Na-phosphate pH 6.0 with the same system. Then, the sample was loaded onto a SP Sepharose Fast Flow (Amersham Biosciences) column equilibrated with the phosphate buffer. After washing, the enzyme was eluted at a flow rate of 1 ml min^{-1} with a linear gradient from 0 to 0.4 M NaCl. The active fractions were pooled, concentrated and desalted on an Amicon PM-30 membrane.

1.3.5 Protein determination

Protein concentration was determined using the BioRad Protein Assay (BioRad), with BSA as standard.

1.3.6 Reduction and alkylation of cysteine residues

Mass spectrometry analyses were performed on the Comassie blue-stained proteins (0.4 nmol) excised from a preparative SDS electrophoresis on a 9% polyacrylamide gel. Excised bands were washed with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced, by incubation in 10 mM dithiothreitol for 45

min at 56°C, and carboxamidomethylated by using 55 mM iodoacetamide in 0.1 M NH_4HCO_3 for 30 min, in the dark, under nitrogen atmosphere at room temperature.

Mass spectrometry analyses were also performed on the soluble recombinant proteins (1 nmol) reduced in Tris 300 mM pH 8.0, EDTA 20 mM (containing guanidinium chloride 6 M) by incubation with a 10:1 molar excess of dithiothreitol over the protein SH groups at 37°C for 2h. The free cysteine residues were alkylated by using a 5:1 excess of iodoacetamide over the total SH groups at room temperature for 1h in the dark under a nitrogen atmosphere. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD10 pre-packed column (Pharmacia Biotech), equilibrated and eluted in 0.4% ammonium bicarbonate, pH 8.5.

1.3.7 Enzymatic hydrolysis

The gel particles were washed with ammonium bicarbonate and acetonitrile. Enzymatic digestions were carried out with trypsin (10 μg ml^{-1}) in 50 mM ammonium bicarbonate, pH 8.5 at 4°C for 2h. The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 18h at 37°C. Minimum reaction volume, sufficient for complete rehydratation of the gel, was used. Peptides were then extracted, washing the gel particles with 20 mM ammonium bicarbonate and 0.1% trifluoroacetic acid in 50% acetonitrile at room temperature and then lyophilised. Aliquots of the digests were directly analysed by MALDI-MS (Matrix Assisted Laser Desorption Ionisations Mass Spectrometry). The tryptic peptide mixture was deglycosylated with peptide N-glycosidase F (PNGaseF) by incubation overnight in 0.4% ammonium bicarbonate, pH 8.5 at 37°C. Aliquots of the digest were analysed directly by MALDI MS. V8, trypsin and AspN digestions in solution were performed on carboxamidomethylated laccase samples in 0.4% ammonium bicarbonate, pH 8.5 at 37°C for 18h using an enzyme/substrate ratio of 1:50.

1.3.8 Mass spectrometry analysis

MALDI mass spectra were recorded using a Voyager DE and Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems). A mixture of analyte solution, α-cyano-4-hydroxy-cinnamic acid or di-hydroxy-benzoic as matrices, bovine insuline and horse heart myoglobin as standards were applied to the sample plate and air-dried. Mass calibration was obtained using the quasi-molecular ions (MH^+) from horse myoglobin (16,952.50 *m/z*), bovine insuline (5734.59 *m/z*), and α-cyano-4-hydroxy-cinnamic acid (379.06 *m/z*) as internal standards. Raw data were analysed by using computer software provided by the manufacturer and are reported as average or monoisotopic masses.

2 RESULTS

2.1 *Recombinant expression in* Kluyveromyces lactis

In this section it is reported on the heterologous expression of two laccase isoenzymes secreted by *P. ostreatus*: POXC (Palmieri *et al.*, 1993) and POXA1b (Giardina *et al.*, 1999), in the budding yeast *K. lactis*.

2.1.1 Recombinant laccases production

2.1.1.1 Codon usage

Every host species has its own set of biases in the codon usage, and tRNA population closely matched to the overall codon bias of the resident mRNA population. A preliminary study of the codon usage of both organisms used for the expression (the host for the heterologous expression, *K. lactis*, and the source of the cDNAs, *P. ostreatus*) is necessary as drastic differences in the codon usages could interfere with the correct translation of the mRNA giving rise to substitutions in the polypeptide sequence, and/or to incomplete proteins. The study was performed using the software available on the website: http//www.kazusa.or.jp/codon/. From the comparison of the codon usage of the two organisms it is possible to note that differences are not such drastic to prevent the correct translation of the proteins. In table 2.1 the frequency of codon usage is expressed per thousand codons.

2.1.1.2 Vector construction

The plasmid used for the heterologous expression (pYG132), is a shuttle vector containing both a *K. lactis* and an *E. coli* origin of replication, a β-Lactamase (*bla*) and an amino glycoside phosphotransferase (*aph*) genes conferring ampicillin and geneticin (G418) resistance in bacterial and yeast cells, respectively and finally a *K. lactis* ethanol-inducible *KlADH4* promoter and *S. cerevisiae* phosphoglycerate kinase (*PGK*) terminator controlling the expression of inserts. Foreign cDNAs can be inserted into pYG132 plasmid at a unique HindIII site. In order to introduce an appropriate HindIII restriction site into the laccase encoding sequences, the 5' regions of both cDNAs were amplified with Fwpoxc/Revpoxc and Fwpoxa1b/Revpoxa1b primers, using pUC13 containing POXC cDNA (Cpox13) and pUC18 containing POXA1b cDNA (A1bpox18) respectively as templates. The HindIII DNA fragments containing the laccase encoding sequences, including their signal peptides, were ligated into the expression vector. Cloning strategies are shown in the figure 2.1.

The sequence of the amplified *poxc* cDNA cloned into the expression vector exactly matched the deposited sequence, whilst DNA sequencing of the *poxa1b* clone, as well as of three other clones tested, revealed one nucleotide deviating from the deposited sequence in position 687. This mutation generates one amino acid substitution, involving residue 201 of the mature protein (codon GAC instead of AAC, coding D instead of N).

A third expression vector, in which the native *poxc* signal was replaced with *K. lactis* killer toxin (α-subunit) pre-sequence (Stark and Boyd, 1986), was constructed. A synthetic DNA fragment corresponding to the pre-sequence (16 aa) of the *K. lactis* killer toxin (α-subunit) was opportunely ligated to the DNA fragment carrying the *poxc* mature cDNA. A HindIII fragment containing the sequence coding for the *K. lactis* killer toxin

pre-sequence in frame with the *poxc* cDNA was subcloned into pYG132. Figure 2.2 shows the cloning strategy exploited to obtain the pYCtk vector.

Table2.1 Codon usage table.

Codon	*K. lactis*	*P. ostreatus*	Codon	*K. lactis*	*P. ostreatus*	Codon	*K. lactis*	*P. ostreatus*
UUU (F)	19,7	7,3	CAA (Q)	29,4	17,9	GCU (A)	28	30,1
UUC (F)	23,4	36,3	CAG (Q)	11,7	17	GCC (A)	11,6	31,7
UUA (L)	23,6	3,3	AAU (N)	31,9	15,6	GCA (A)	15,5	14
UUG (L)	33,6	16,5	AAC (N)	25,6	35,9	GCG (A)	4,9	18,2
CUU (L)	11,8	19,5	AAA (K)	37,6	9,4	UGU (C)	9,5	5,8
CUC (L)	4,3	27,6	AAG (K)	33,8	21,1	UGC (C)	3,1	17,2
CUA (L)	11,7	2,9	GAU (D)	41,2	24,7	UGA (stop)	0,6	0,7
CUG (L)	5,2	14,3	GAC (D)	18,4	36,5	UGG (W)	11,4	10,5
AUU (I)	27,6	21,4	GAA (E)	47,8	17,5	CGU (R)	6,6	8,1
AUC (I)	20,7	37,3	GAG (E)	15,8	20,2	CGC (R)	1,5	12,4
AUA (I)	16,1	3,7	UCU (S)	26,9	12,6	CGA (R)	2,2	4,4
AUG (M)	20,9	16,6	UCC (S)	12,5	17	CGG (R)	1,5	3
GUU (V)	25,7	19,8	UCA (S)	16,4	6,3	AGU (S)	12,9	8,9
GUC (V)	14,2	32	UCG (S)	7,7	19,4	AGC (S)	6,3	12,3
GUA (V)	10,9	5,7	CCU (P)	13,3	21,2	AGA (S)	24,7	3,6
GUG (V)	10,8	12,7	CCC (P)	3,7	21,5	AGG (S)	6	4,8
UAU (Y)	20,5	8,8	CCA (P)	21,2	16,2	GGU (G)	35,3	20,4
UAC (Y)	17,2	13	CCG (P)	4,1	8,3	GGC (G)	5,5	29,6
UAA (stop)	1,1	2,2	ACU (T)	22,9	20,1	GGA (G)	11,5	16,5
UAG (stop)	0,4	0,4	ACC (T)	12,8	25	GGG (G)	5,2	12,2
CAU (H)	14,2	11,9	ACA (T)	15	9			
CAC (H)	6,9	13,2	ACG (T)	5,8	16,9			

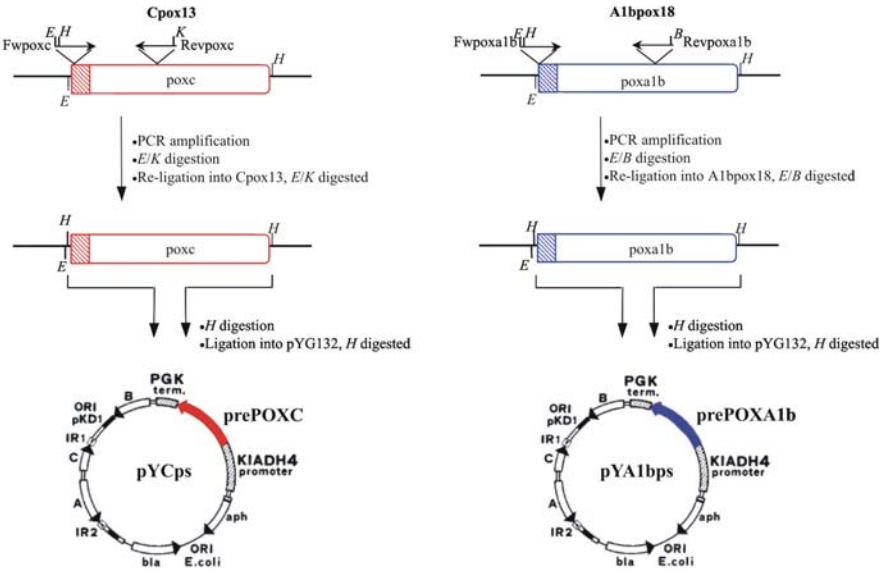

Fig. 2.1Construction of pYCps and pYA1bps expression vectors. The hatched regions encode the respective signal peptides. Fwpoxc and Revpoxc, and Fwpoxa1b and Revpoxa1b primers were used to amplify laccase cDNAs and to insert a HindIII site. A, B, C, IR1 and IR2 are sequences of the original pKD1 plasmid. β-Lactamase (*bla*) and aminoglycoside phosphotransferase (*aph*) genes are also indicated. *H*, HindIII; *E*, EcoRI; *K*, KpnI; *B*, BglII.

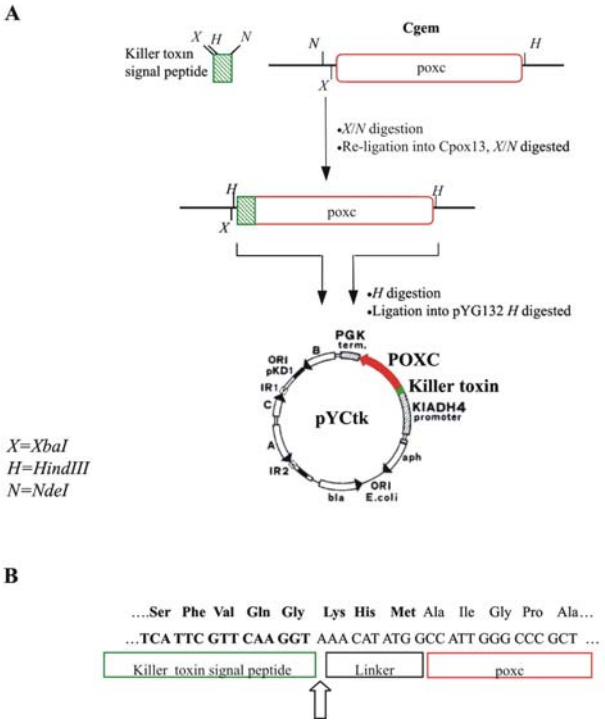

Fig. 2.2 (A) Construction of the expression vector pYCtk. The green hatched region encodes the K. lactis killer toxin signal peptide. H, HindIII; N, NdeI; X, XbaI. (B) Junctions between K. lactis killer toxin secretion signal, linker, and the N-terminal sequence of mature POXC. The hypothetical cleavage sites, presumed to be recognized by the *K. lactis* signal peptidase, is shown as open arrow.

2.1.1.3 Plate assay and clones screening

The production of recombinant laccases was assayed on plate using ABTS as substrate. Several transformants (≈85%) carrying pYA1bps vector gave colour change after 3 days, whilst no colour change was observed either for transformants carrying the pYCps or the pYCtk vectors, both containing the *poxc* cDNA (Fig. 2.3).

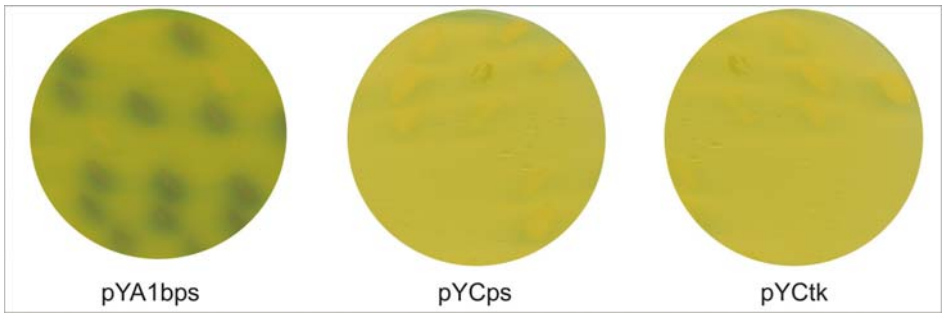

Fig. 2.3 Plate assay of *K. lactis* transformants.

30 different clones from each transformation were grown in liquid cultures and their laccase production was tested. The amount of extracellular laccase activity produced after 3 days by the pYA1bps transformants ranged from 0 to $9x10^{-2}$ U ml^{-1}. On the other hand, pYCps transformants produced very low activity, detectable only on 10-fold concentrated samples, ranging from 0 to $6x10^{-4}$ U ml^{-1}. No laccase activity and no positive signal on western blot analyses of cellular extracts and culture supernatants were detected, either in pYCtk transformants or in control cultures.

The best producing clones (HpYC for r-POXC and HpYA1b for r-POXA1b) were selected to study the time course of laccase production and to characterise the recombinant enzymes. Western blot analyses were conducted on cellular extracts and culture supernatants from the best recombinant yeasts (Fig. 2.4). Considering that the culture supernatant volumes loaded on the SDS-PAGE corresponded to an amount of cells 3-fold higher than that used for the cellular extracts, the recombinant proteins seem to be distributed almost equally in the two compartments.

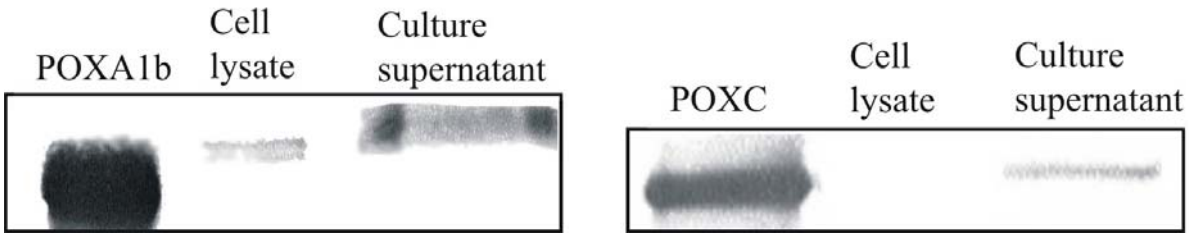

Fig. 2.4 Western Blotting analysis of cellular extracts and culture supernatants of the laccase positive yeasts expressing rPOXA1b and rPOXC.

The protein recognised by the anti-POXA1b antibodies exhibits a slightly higher apparent molecular mass (Mr) than that of the native enzyme, whilst the protein

recognised by the anti-POXC antibodies has a Mr very closed to that of the native laccase.

2.1.1.4 Effect of growth conditions on laccase production

The best producing clones (HpYC for r-POXC and HpYA1b for r-POXA1b) were selected to study the time course of laccase production and to characterise the recombinant enzymes. Cultures were carried out to improve laccase production varying growth conditions, with a particular attention to the effect of inducer, nutrients and copper concentration. The selected transformants were first grown in YPPD geneticin medium in the presence of 0.1 mM $CuSO_4$ with variable ethanol (inducer) concentration. Both HpYC and HpYA1b produced the highest laccase activity (0.007 and 2.03 U ml^{-1} respectively) in the presence of 0.5% ethanol. Activities normalised to the culture growth (mU/OD_{600}) are reported in the figure 2.5. The presence of 2% ethanol reduced the cell growth of 50% after 4 days.

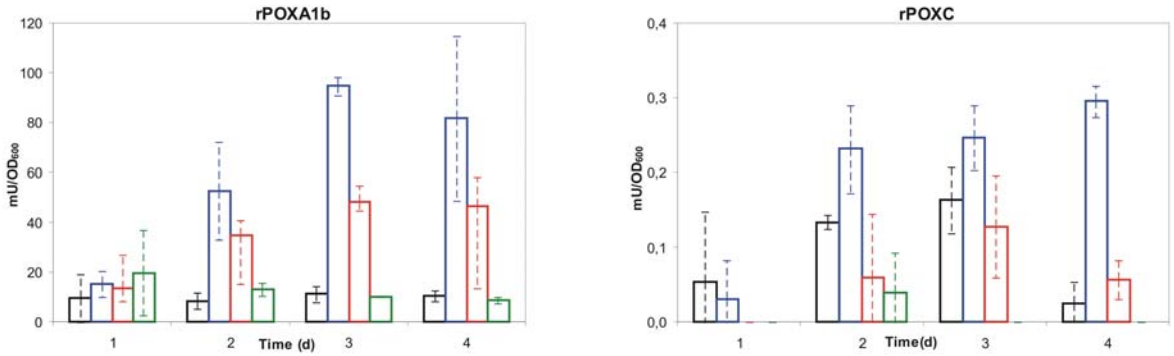

Fig. 2.5 Specific production of rPOXA1b and rPOXC. Ethanol effect on laccase secretion. Cells were grown in YPPD selective medium containing 0.1 mM $CuSO_4$ and different ethanol concentration. □, 0% EtOH; □ 0.5%EtOH; □, 1%EtOH; □, 2%EtOH.

The influence of media composition on laccase production was then evaluated in the presence of 0.5% ethanol and 0.1 mM $CuSO_4$. HpYC and HpYA1b were cultivated in different rich media containing glucose, galactose or glucose and galactose. The highest secreted laccase activities (0.067 U ml^{-1} and 3.9 U ml^{-1} respectively) were obtained in YPPG medium (Fig. 2.6).

Copper concentration was then varied in the YPPG geneticin 0.5% ethanol medium and the activity of the recombinant proteins after three days of growth were plotted *versus* copper concentration (Fig. 2.7). rPOXC activity was detected in the medium even in absence of copper, but laccase activity increased as copper concentration increased. A concentration of at least 0.5 mM $CuSO_4$ was required to produce the highest rPOXC activity (0.09 U ml^{-1}). On the other hand, rPOXA1b activity appeared not to be strictly dependent on copper concentration, as there was not any noticeable improvement in the secreted activity (4.2 U ml^{-1}) with the increase in copper concentration.

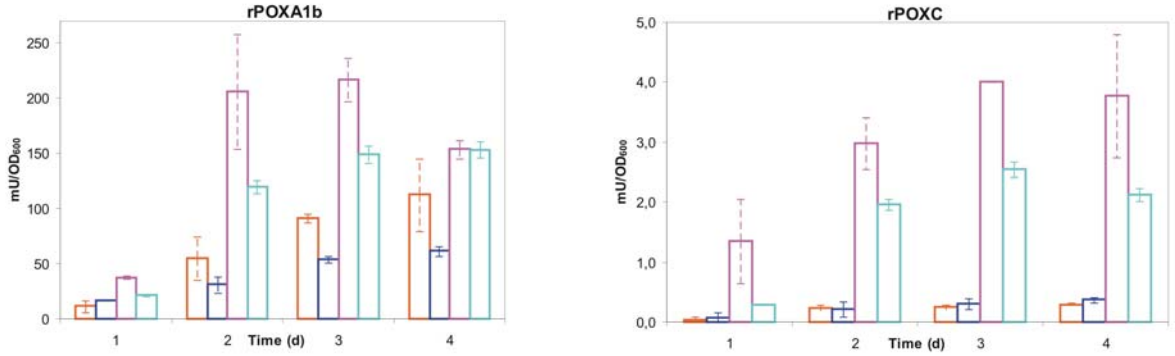

Fig. 2.6 Specific production of rPOXA1b and rPOXC. Nutrient effect on the heterologous production of recombinant laccases. Cells were grown in selective medium supplemented with 0.5% ethanol and 0.1mM $CuSO_4$. □, YPD (10 g l^{-1} yeast extract, 20 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); □, YPPD (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); □, YPPG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} galactose); □, YPPDG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose, 20 g l^{-1} galactose).

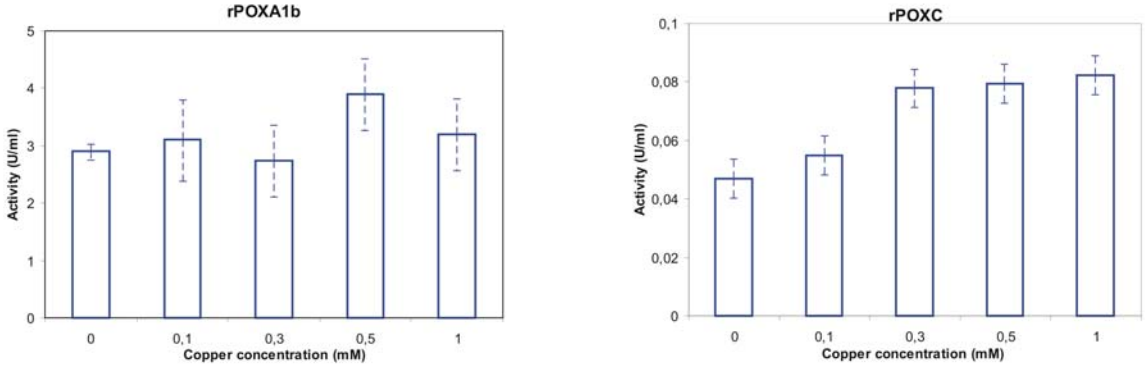

Fig. 2.7 Influence of copper on the secreted activities of recombinant laccases.

Cell growth was not affected by copper, as the biomass yield was almost identical for both transformants at all copper concentrations. No variation in the amount of produced enzymes was observed by Western blot analysis as function of copper concentration (data not shown).

Table 2.2 summarise the results obtained in the heterologous expression of *P. ostreatus* laccases in *K. lactis*: the higher efficiency of rPOXA1b than rPOXC transformants is easily observable.

Table 2.2 *K. lactis* cells secreting recombinant laccases specific production. Data are referred to 3 days culture

	mU/OD_{600}	
Condition	**rPOXA1b**	**rPOXC**
EtOH (%)		
0	11,2	0.16
0.5	94.6	0.25
1	48,2	0.13
2	10,1	##
Medium		
YPPD	94.6	0.25
YPD	54.4	0.30
YPPG	216.9	4.0
YPPDG	149.2	2.5
$CuSO_4$ (mM)		
0	191.0	2.9
0.1	217. 0	4.0
0.3	190.9	6.0
0.5	156.91	5.6
1	247.0	5.9

2.1.2 Recombinant laccases purification and characterisation.

2.1.2.1 rPOXA1b

HpYA1b transformant was grown in 1l of selective YPPG supplemented with 0.5% ethanol and 0.5 mM $CuSO_4$ for 3 days. The enzyme was purified from the culture broth by ammonium sulphate precipitation (80%) and eluted at 0.28 M NaCl from a cation exchange chromatography (Fig. 2.8) (Table 2.3).

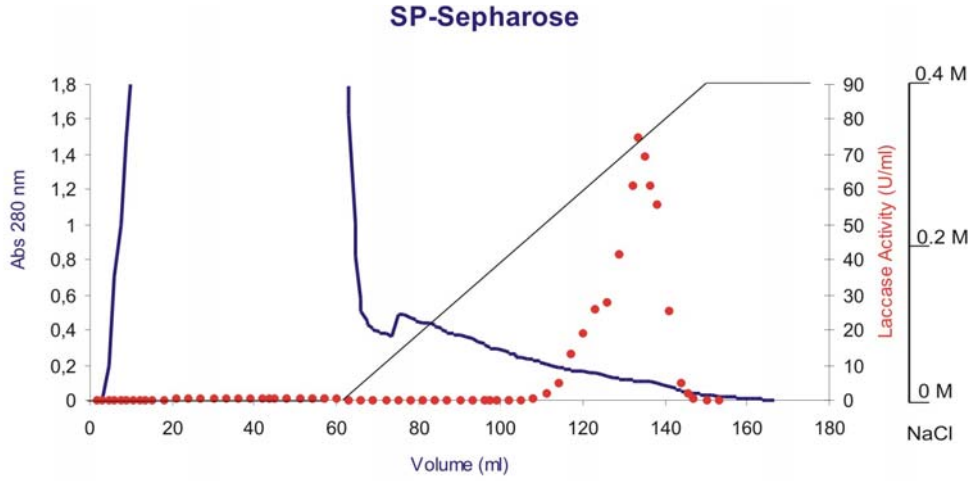

Fig. 2.8 Elution profile from the cation exchange chromatography.

Table 2.3 rPOXA1b purification from *Kluyveromyces lactis* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	1392	94.7	14.7	100	1.0
$(NH_4)_2SO_4$ precipitate	910	32.0	28.4	65.4	1.9
S Sepharose	745	0.6	1307.0	53.5	88.9

The recombinant protein was found to be homogeneous on SDS-PAGE on which it behaved as a 71kDa protein whereas native POXA1b migrates as a 62kDa protein (Giardina *et al.*, 1999) (Fig. 2.9A).

On IEF, the recombinant protein showed two equally represented bands at pH 7.2 and 7.3 whereas the native POXA1b shows several bands in the pH range of 6.5 ÷ 7.6 (Fig. 2.9C).

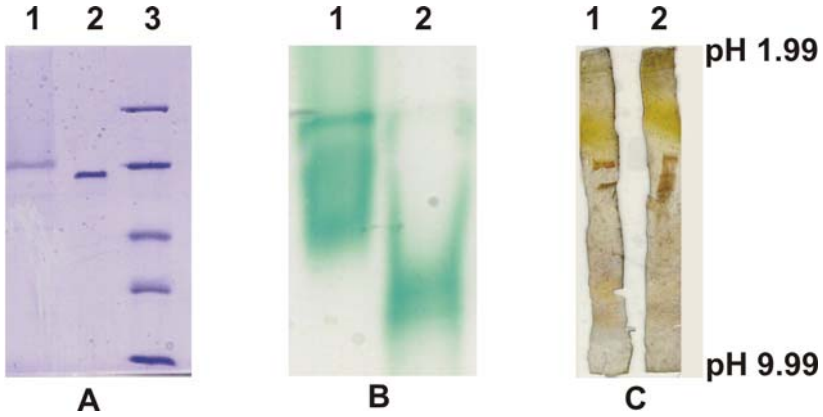

Fig. 2.9 Electrophoretyc analyses of rPOXA1b. Lane 1 recombinant protein; lane 2 native protein; lane 3 molecular weight standards (116 kDa, 66.2 kDa, 45 kDa, 35 kDa, 25 kDa). (A) SDS-PAGE, Comassie staining; (B) NATIVE PAGE, ABTS staining; (C) IEF, Silver staining.

The kinetic constants determined for rPOXA1b are reported in table 2.4. No marked differences between native and recombinant POXA1b were observed when DMP was used as substrate. On the other hand although k_{cat} for ABTS were similar for the two proteins, it is worth noting that K_M towards ABTS of the recombinant protein was about one order of magnitude lower than that of the native protein.

Table 2.4 Kinetic constants of native and recombinant POXA1b.

Substrate	K_M (mM)		k_{cat} (min^{-1})	
	POXA1b	rPOXA1b	POXA1b	rPOXA1b
ABTS	0.47±0.06	0.037±0.005	$(9.0\pm1.7)x10^4$	$(9.0\pm1.2)x10^4$
DMP	0.26±0.09	0.29±0.04	$(1.5\pm0.1)x10^4$	$(5.5\pm0.7)x10^4$

Mass spectrometry analyses of rPOXA1b allowed validating 83% of the total amino acid sequence (Fig. 2.10). The signal peptide is correctly cleaved, whilst C-terminus was mapped as peptide 497-511 (1536.90 m/z), indicating that two amino acids from the C-terminus were cleaved. rPOXA1b amino acid sequence contains five potential N-glycosylation sites (N-X-S/T): three of them (N294, N434 and N470) were found glycosylated in *K. lactis*. The oligosaccharide chains linked to the pentasaccharide core ranges from five to eight mannose residues for N294, from one to ten mannose residues for N434, and from zero to four mannose residues for N470. No information was found regarding the residue N342, whilst N490 was found not modified. Native protein shows N294, N470 and N490 with up to five mannose residues linked to the pentasaccharide core, N434 not modified, whilst no information was available regarding residue N342 (Giardina *et al.*, 1999).

pH and temperature (60°C) stability of rPOXA1b were determined (Table 2.5). The performances of the recombinant laccase were found similar to those of the native one.

POXA1b

```
SIGPRGTLNI ANKVIQPDGF YRSTVLAGGS YPGPLIKGKT GDRFQINVVN 50

KLADTSMPVD TSIHWHGLFV KGHNWADGPA MVTQCPIVPG HSFLYDFEVP 100

DQAGTFWYHS HLGTQYCDGL RGPLVVYSKN DPHKRLYDVD DESTVLTVGD 150

WYHAPSLSLT GVPHPDSTLF NGLGRSLNGP ASPLYVMNVV KGKRYRIRLI 200

DTSCDSNYQF SIDGHTFTVI EADGENTQPL QVDQVQIFAG QRYSLVLNAN 250
                                                  *
QAVGNYWIRA NPNSGDPGFE NQMNSAILRY KGARSIDPTT PEQNATNPLH 300

EYNLRPLIKK PAPGKPFPGG ADHNINLNFA FDPATALFTA NNHTFVPPTV 350

PVLLQILSGT RDAHDLAPAG SIYDIKLGDV VEITMPALVF AGPHPIHLHG 400
                                    *
HTFAVVRSAG SSTYNYENPV RRDVVSIGDD PTDNVTIRFV ADNAGPWFLH 450
                    *
CHIDWHLDLG FAVVFAEGVN QTAAANPVPE AWNNLCPIYN SSNPSKLLMG 500

TNAIGRLPAP LKA                                        513
```

Fig. 2.10 Amino acid sequence of POXA1b. The underlined stretches have been verified by MALDI MS for the recombinant protein. Putative N-glycosylation sites are in bold; sites found glycosylated are indicated by an asterisk.

Table 2.5 Stability of recombinant laccases at 60°C and at different pHs.

Condition	Stability ($t_{1/2}$)	
	POXA1b	rPOXA1b
60°C	3.0 h	4.2 h
pH3	0.8 d	3.5 d
pH5	6.0 d	6.4 d
pH7	7.0 d	16.6 d
pH10	100 d	27.3 d

2.1.2.2 rPOXC

The recombinant enzyme was purified from the culture broth of HpYC transformant grown in the same conditions as those described for HpYA1b. Ammonium sulphate precipitation, hydrophobic interaction chromatography (Fig. 2.11 A), and anionic exchange chromatography (Fig. 2.11 B) were sequentially used (Table 2.6).

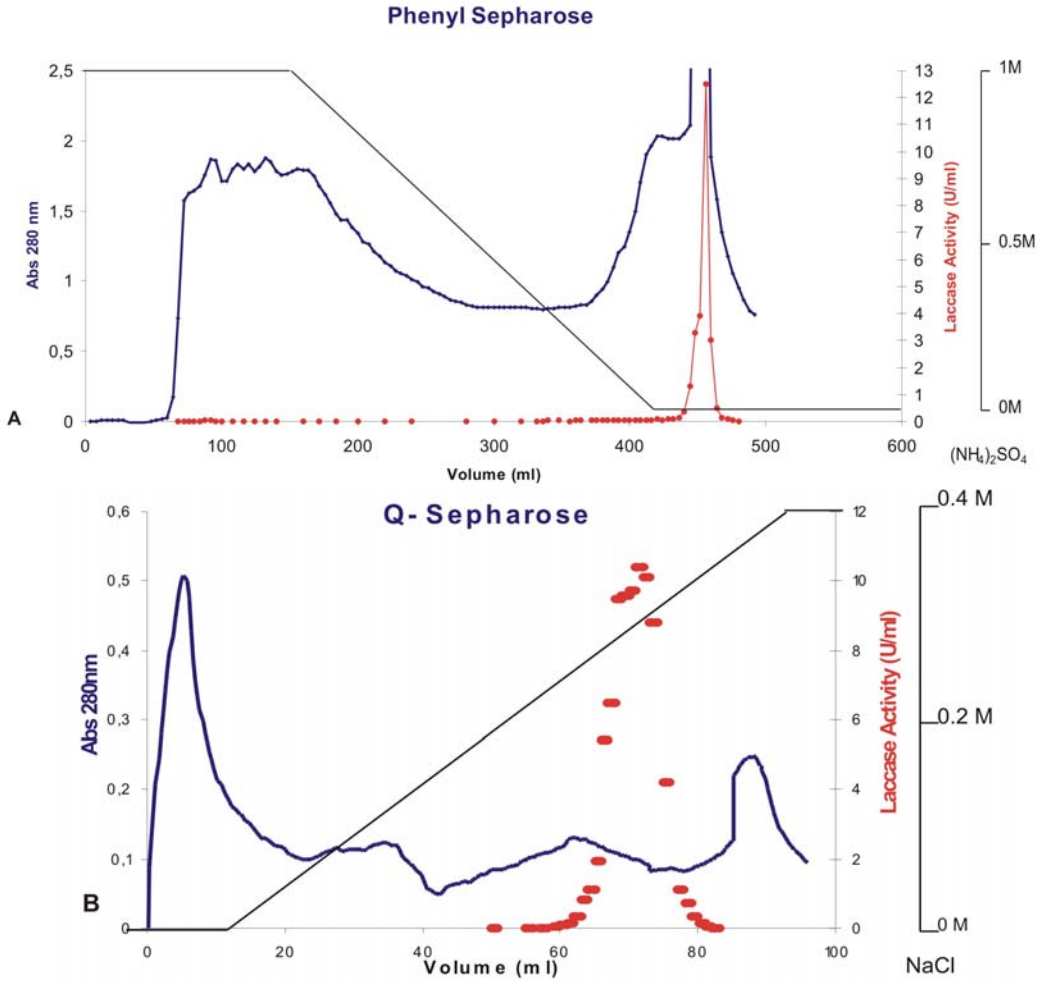

Fig. 2.11 Chromatograms of rPOXC purification. (A) hydrophobic interaction chromatography; (B) anionic exchange chromatography.

The active fraction eluted at 0.28 M NaCl from Q-Sepharose chromatography appeared to be homogeneous on SDS-PAGE and the recombinant protein migrated with an apparent Mr similar to that of native POXC (Fig. 2.12 A). Recombinant and native proteins displayed the same mobility when analysed by native PAGE (Fig. 2.12 B) and IEF.

Table 2.6 rPOXC purification from *Kluyveromyces lactis* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	54.6	106.0	0.51	---	1.0
$(NH_4)_2SO_4$ precipitate	73.2	100.8	0.73	100	1.4
Phenyl Sepharose	59.3	3.8	15.6	81.1	30.6
Q Sepharose	59.1	1.1	51.8	80.7	101.6

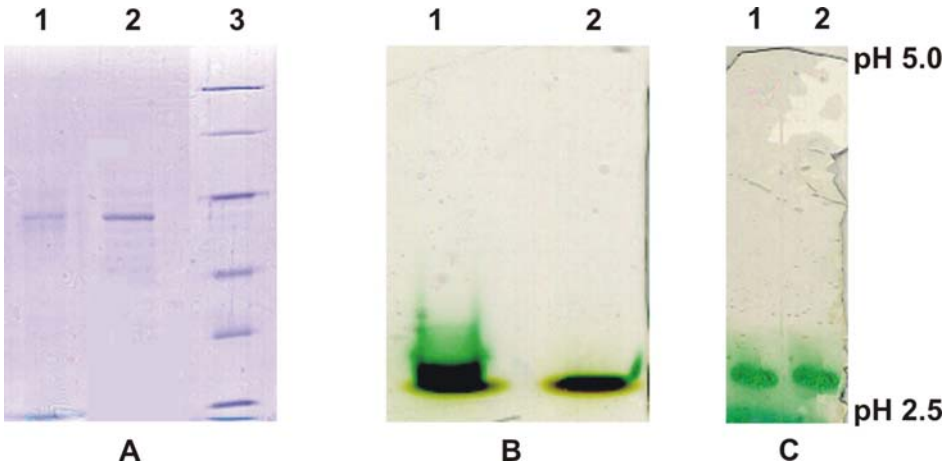

Fig. 2.12 Electrophoretyc analyses of rPOXC. Lane 1 recombinant protein; lane 2 native protein; lane 3 molecular weight standards (116 kDa, 66.2 kDa, 45 kDa, 35 kDa, 25 kDa). (A) SDS-PAGE, Comassie staining; (B) NATIVE PAGE, ABTS staining; (C) IEF, ABTS staining.

Kinetic constants determined for rPOXC are reported in table 2.7. As earlier observed for rPOXA1b, rPOXC K_M towards ABTS is about one order of magnitude lower than that of the native protein, whilst K_M towards DMP is similar. On the other hand, k_{cat} is significantly lower than that of the native enzyme, with the two substrates tested.

No variation of specific activity was observed after incubation of rPOXC at room temperature with copper using either 5 mM $CuSO_4$ or 5mM $CuSO_4$ in presence of 0.1, 0.5, 1 mM sodium ascorbate as Larrondo. *et al.*, (2003) observed with a *Ceriporiopsis* laccase.

Mass spectrometry analyses of rPOXC allowed the validation of 70% of the amino acidic sequence (Fig. 2.13). rPOXC signal peptide is correctly cleaved, but no information regarding C-terminal sequence could be obtained. POXC amino acidic sequence contains two potential N-glycosylation sites, and one of them (N444) was found glycosylated in *K. lactis*. The oligosaccharide chains linked to the pentasaccharide core ranges from three to seven mannose residues. In the native protein the same site was

found glycosylated with up to six mannose residues linked to the pentasaccharide core (Giardina *et al.*, 1996).

Table 2.7 rPOXC Kinetic constants.

Substrate	K_M (mM)		k_{cat} (min^{-1})	
	POXC	**rPOXC**	**POXC**	**rPOXC**
ABTS	0.28±0.04	0.029±0.004	$(1.4\pm0.2)x10^5$	$(3.2\pm0.4)x10^3$
DMP	0.07±0.01	0.13±0.02	$(4.8\pm0.2)x10^5$	$(1.0\pm0.1)x10^3$

POXC

AIGPAGNMYI VNEDVSPDGF ARSAVVARSV PATDPTPATA SIPGVLVQGN 50

KGDNFQLNVV NQLSDTTMLK TTSIHWHGFF QAGSSWADGP AFVTQCPVAS 100

GDSFLYNFNV PDQAGTFWYH SHLSTQYCDG LRGPFVVYDP SDPHLSLYDI 150

DNADTVITLE DWYHIVAPQN AAIPTPDSTL INGKGRYAGG PTSPLAIINV 200

ESNKRYRFRL VSMSCDP**N**FT FSIDGHSLLV IEADAVNIVP ITVDSIQIFA 250

GQRYSFVLTA NQAVDNYWIR ANPNLGSTGF VGGINSAILR YAGATEDDPT 300

TTSSTSTPLL ETNLVPLENP GAPGPPVPGG ADININLAMA FDFTTFELTI 350

NGVPFLPPTA PVLLQILSGA STAASLLPSG SIYELEANKV VEISMPALAV 400
*

GGPHPFHLHG HTFDVIRSAG STTYNFDTPA RRDVVNTGTG AND**N**VTIRFV 450

TDNPGPWFLH CHIDWHLEIG LAVVFAEDVT SISAPPAAWD DLCPIYNALS 500

DNDKGGIVPS 510

Fig. 2.13 Amino acid sequence of POXC. The underlined stretches have been verified by MALDI MS for the recombinant protein. Putative N-glycosylation sites are in bold; site found glycosylated is indicated by an asterisk.

pH and temperature (60°C) stability of recombinant proteins were determined (Table 2.8). Except for the stability at pH 5 and 7, where the recombinant enzyme was proved to be more stable than the native enzyme, the performances of the recombinant laccase were found similar to those of the native one.

Table 2.8 Stability of recombinant laccases at 60°C and at different pHs.

Condition	Stability ($t_{1/2}$)	
	POXC	**rPOXC**
60°C	0.6 h	1.3 h
pH3	0.3 h	1.7 h
pH5	7.4 h	1.8 d
pH7	7.0 h	8.0 d
pH10	n. d.	4.2 d

2.2 *Recombinant expression in* Saccharomyces cerevisiae

The heterologous expression in the yeast *S. cerevisiae* of the two *P. ostreatus* laccase isoenzymes POXC (Palmieri *et al.*, 1993) and POXA1b (Giardina *et al.*, 1999), is here described.

2.2.1 Recombinant laccases production

2.2.1.1 Codon usage

The study of the codon usage was also performed for *S. cerevisiae*. From the comparison of the codon usage of the two organisms it is possible to note that the differences present should probably not interfere with the correct translation of the proteins. In table 2.9 the frequency of codon usage is expressed per thousand codons.

Table 2.9 Codon usage table.

Codon	*S. cerevisiae*	*P ostreatus*	*Codon*	*S. cerevisiae*	*P. ostreatus*	*Codon*	*S. cerevisiae*	*P. ostreatus*
UUU (F)	26,1	7,3	CAA (Q)	27,5	17,9	GCU (A)	21,1	30,1
UUC (F)	18,2	36,3	CAG (Q)	12,1	17	GCC (A)	12,5	31,7
UUA (L)	26,4	3,3	AAU (N)	36,1	15,6	GCA (A)	16,2	14
UUG (L)	27,1	16,5	AAC (N)	24,9	35,9	GCG (A)	6,1	18,2
CUU (L)	12,2	19,5	AAA (K)	42,1	9,4	UGU (C)	8	5,8
CUC (L)	5,4	27,6	AAG (K)	30,7	21,1	UGC (C)	4,7	17,2
CUA (L)	13,4	2,9	GAU (D)	37,8	24,7	UGA (stop)	0,6	0,7
CUG (L)	10,4	14,3	GAC (D)	20,2	36,5	UGG (W)	10,3	10,5
AUU (I)	30,2	21,4	GAA (E)	45,9	17,5	CGU (R)	6,5	8,1
AUC (I)	17,1	37,3	GAG (E)	19,1	20,2	CGC (R)	2,6	12,4
AUA (I)	17,8	3,7	UCU (S)	23,6	12,6	CGA (R)	3	4,4
AUG (M)	20,9	16,6	UCC (S)	14,3	17	CGG (R)	1,7	3
GUU (V)	22	19,8	UCA (S)	18,8	6,3	AGU (S)	14,2	8,9
GUC (V)	11,6	32	UCG (S)	8,6	19,4	AGC (S)	9,7	12,3
GUA (V)	11,8	5,7	CCU (P)	13,6	21,2	AGA (S)	21,3	3,6
GUG (V)	10,7	12,7	CCC (P)	6,8	21,5	AGG (S)	9,2	4,8
UAU (Y)	18,8	8,8	CCA (P)	18,2	16,2	GGU (G)	23,9	20,4
UAC (Y)	14,7	13	CCG (P)	5,3	8,3	GGC (G)	9,7	29,6
UAA (stop)	1	2,2	ACU (T)	20,3	20,1	GGA (G)	10,9	16,5
UAG (stop)	0,5	0,4	ACC (T)	12,6	25	GGG (G)	6	12,2
CAU (H)	13,7	11,9	ACA (T)	17,8	9			
CAC (H)	7,8	13,2	ACG (T)	7,9	16,9			

2.2.1.2 Vector construction

Plasmids used for the heterologous expression are shuttle vectors containing sequences for the propagation and selection both in bacterial and in yeast cells, and sequence for the expression of a gene cloned in it (Table 2.10).

Table 2.10 Characteristics of expression vectors.

Vector	2µ	CEN/ARS	Selection Bacteria/yeasts	Promoter	Terminator	Leader sequence
pAC1	-	+	Amp/URA	*ADH1*	*CYC1*	Laccase
pSAL4	+	-	Amp/URA	*CUP1*	*CYC1*	Laccase
YEp352	+	-	Amp/URA	*PGK*	-	SUC2

Two yeast replication origins were tested: a 2µ sequence allowing to maintain a high copy number of plasmid per cell, and a CEN/ARS sequence allowing to keep a low copy number of plasmid per cell. Two kinds of promoters were used: constitutive promoter sequences from either the alcohol dehydrogenase I (*ADHI*) gene or from the phosphoglycerate kinase (*PGK*) gene, and the inducible promoter sequence from the metallothionein gene (*CUP1*). The terminator sequence used comes from the *CYC1* gene, coding for the cythocrome C. Two sequence encoding leader peptides were tested, from the sequence encoding the native laccase leader peptide or the sequence encoding the *S. cerevisiae* invertase leader peptide (from the *SUC2* gene).

A unique EcoRI site was used to clone laccase cDNA into pAC1 and pSAL4 plasmids. The 3' regions of both laccase cDNAs were therefore engineered through amplification with FwEcoRV/Crev and FwBglII/A1brev primers, respectively from a pUC13 containing POXC cDNA (Cpox13) and pUC18 containing POXA1b cDNA (A1bpox18 previously prepared) templates. The EcoRI flanked laccase encoding sequences, including their signal peptides, were ligated into pAC1 and pSAL4 respectively under the control of the *ADHI* and *CUP1* promoter regions. Cloning strategies are shown in the figure 2.14.

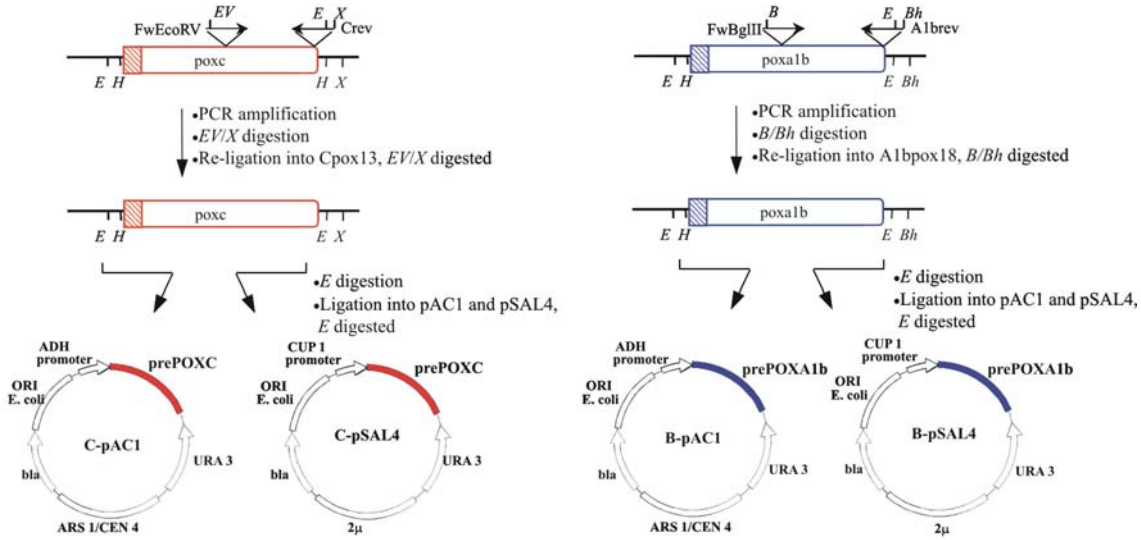

Fig. 2.14 Construction of expression vectors. The hatched regions encode the respective signal peptides. FwEcoRV/Crev and FwBglII/A1brev are the primers used to amplify laccase cDNAs and to insert the EcoRI site. Selection markers, promoter regions and replication origins are indicated. *H*, HindIII; *E*, EcoRI; *X*, XbaI; *EV*, EcoRV; *B*, BglII; *Bh*, BamhI.

Poxc, and *poxa1b* sequences were re-engineered through the replacement of respective endogenous signal peptide coding sequences with the sequence driving the secretion of

invertase in yeast (a 60bp fragment encoding the signal sequence of the *SUC2* gene product) and cloned under the control of the *PGK1* expression cassette, in a vector body derived from YEp 352 (Hill *et al.*, 1986). Cloning strategies are shown in the figure 2.15.

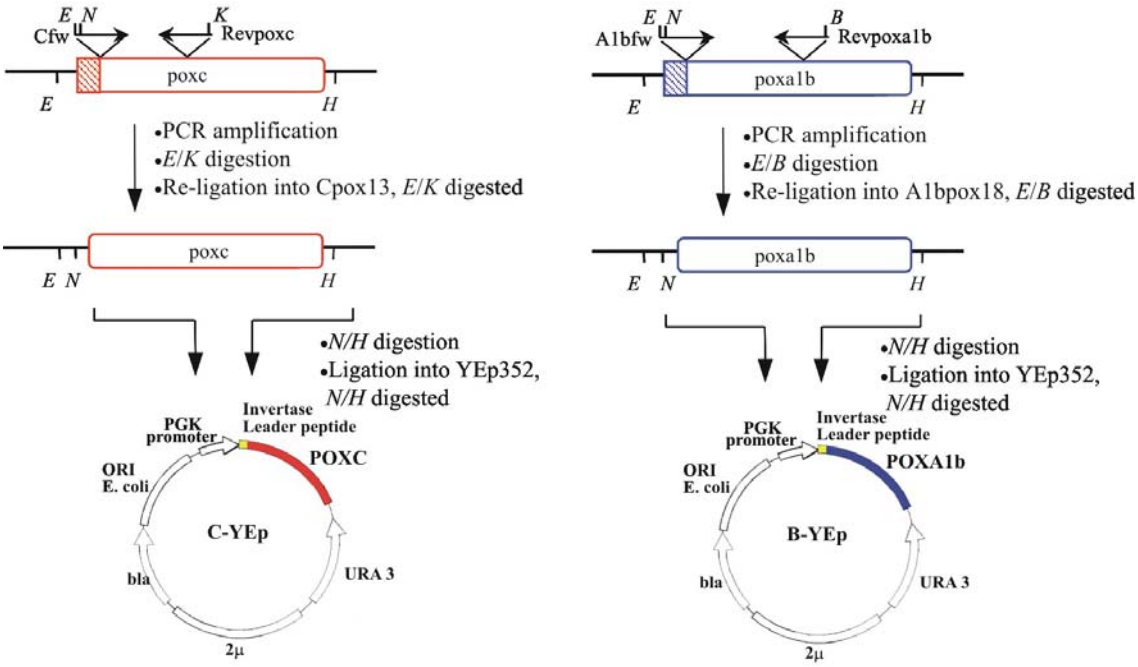

Fig. 2.15 Construction of expression vectors. The yellow regions encode the *S. cerevisiae* invertase signal peptide. Cfw/Revpoxc and A1bfw/Revpoxa1b are the primers used to amplify laccase cDNAs. Selection markers, promoter regions and replication origins are indicated. *H*, HindIII; *E*, EcoRI; *K*, KpnI; *N*, NsiI; *B*, BglII.

The sequence of the amplified *poxc* cDNA cloned into the expression vectors exactly matched the deposited sequence, whilst *poxa1b* clone presents one nucleotide deviating from the deposited sequence in position 687. This mutation generates one amino acid substitution, involving residue 201 of the mature protein (codon GAC instead of AAC, coding D instead of N).

2.2.1.3 Plate assay and clones screening

Production of recombinant laccases was first assayed on plate using ABTS as substrate. Out of the three constructions containing the *poxc* cDNA tested, only the yeasts transformed with C-YEp and C-pSAL4 were found positive for secretion of an active laccase. With vectors containing the *poxa1b* cDNA, all the yeasts transformed were found positive for secretion of an active laccase. On the basis of both the intensity of the colour, and the diameter of the halo, there was a marked difference in laccase expression either among the different constructs used or between the two laccases. Table 2.11 reports performances of laccase secreting yeasts. Yeasts transformed with plasmids derived from pSAL4 appeared to be the best producers of both *P. ostreatus* laccases.

Table 2.11 Plate test on laccase secreting yeast.

Plasmid	C-YEp	C-pAC1	C-pSAL4	B-YEp	B-pAC1	B-pSAL4
ABTS plate test	+	-	++	++	+	+++

When a similar analysis was conducted on liquid culture the amount of rPOXA1b secreted activity was always significantly higher than that of rPOXC (Table 2.12). In confirmation of the plate assay, the pSAL4 derived plasmids with a 2μ as replication origin and the homologous laccase signal peptide were found to give the best results for both laccases, and were selected to further investigate recombinant laccase production.

Table 2.12 Analysis of laccase production.

Plasmid	U ml^{-1}	Time (d)
C-YEp	$2,3*10^{-4}$	4
C-pAC1	0	#
C-pSAL4	$5,4*10^{-4}$	3
B-YEp	$9,0*10^{-3}$	8
B-pAC1	$4,4*10^{-3}$	3
B-pSAL4	0,2	7

Western blot analyses were conducted on cellular extracts and culture supernatants from the best recombinant yeasts (Fig. 2.16). Considering that the culture supernatant volumes loaded on the SDS-PAGE corresponded to an amount of cells 5-fold for C-pSAL4 or 10-fold for B-pSAL4 higher than that used for the cellular extracts, the most of the recombinant proteins are located within the cells, a similar trend was also found by Kiiskinen and Saloheimo (2004) for the heterologous expression of a *M. albomyces* laccase.

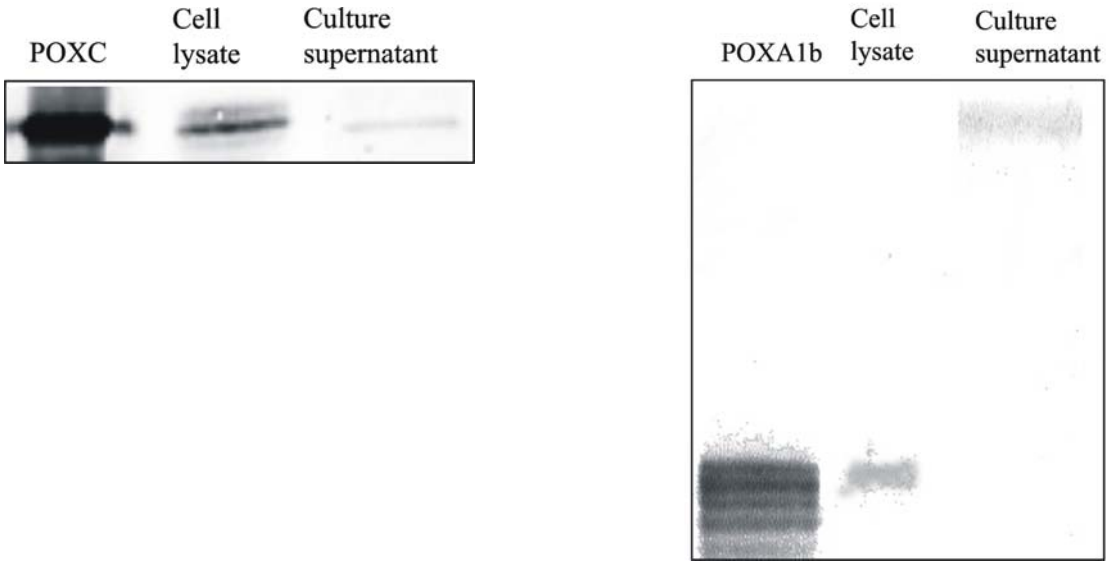

Fig. 2.16 Western Blotting analysis of cellular extracts and culture supernatants of laccase positive yeasts expressing rPOXA1b and rPOXC.

Moreover, secreted rPOXA1b exhibits a higher apparent molecular mass (Mr) than that of the native protein, probably due to the high level of glycosylation, whilst the protein recognised by the anti-POXC antibodies shows a Mr very closed to that of the native laccase. It is worth to note that five putative N-glycosylation sites are present in POXA1b sequence, whilst POXC contains only two putative N-glycosylation sites.

2.2.1.4 Effect of growth conditions on laccase production

Cultures were carried out to improve laccase production varying the growth conditions, with a particular attention to the effect of inducer and nutrients. The promoter used is inducible by copper, which also works as laccase cofactor. Culture supernatants were daily assayed, and results reported as specific production of the recombinant protein (mU/OD_{600}). Selected transformants were first grown in SD medium with variable $CuSO_4$ (inducer) concentrations. Yeast transformed with B-pSAL4 produced the highest laccase activity (0.2 U ml^{-1}) in the presence of 0.6 mM $CuSO_4$. On the other hand, very low laccase activity was detected in cultures of yeasts transformed C-pSAL4 (Fig. 2.17). The cell growth was not affected by copper, as the biomass yield was almost identical for both transformants at all copper concentrations.

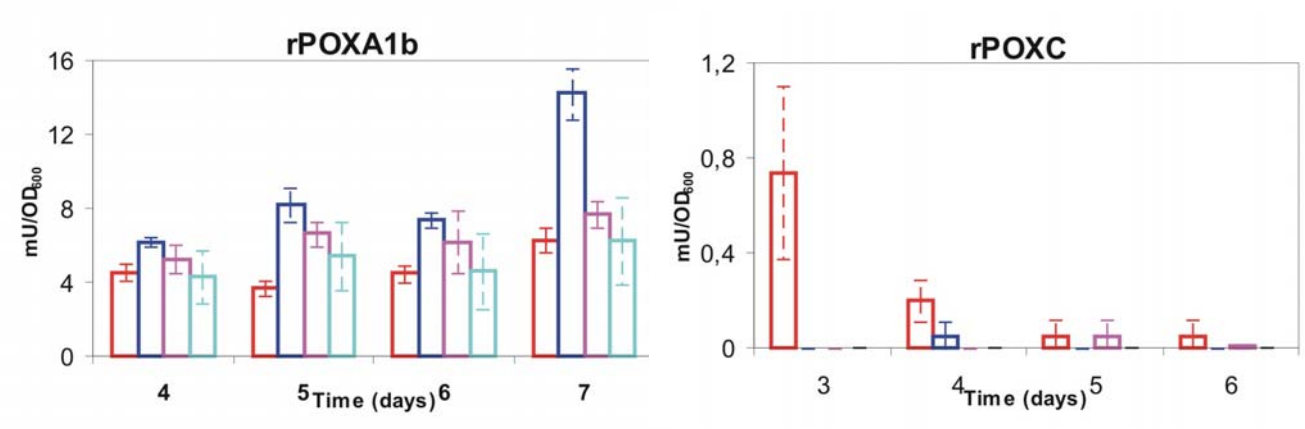

Fig. 2.17 Specific production of rPOXA1b and rPOXC. Inducer (copper) effect on laccase secretion. Cultures were grown in SD medium containing different copper concentration. □, 0.3mM $CuSO_4$; □, 0.6mM $CuSO_4$; □, 1.2mM $CuSO_4$; □, 1.8mM $CuSO_4$.

The influence of media composition on laccase production was then evaluated only for yeast cells transformed with B-pSAL4, the rPOXC secreted activity being too low. B-pSAL4 was cultivated in different selective media containing glucose or galactose in the presence of 0.6 mM $CuSO_4$. The highest activity of secreted recombinant laccase (0.2 U ml^{-1}) was obtained in SD medium (Fig. 2.18).

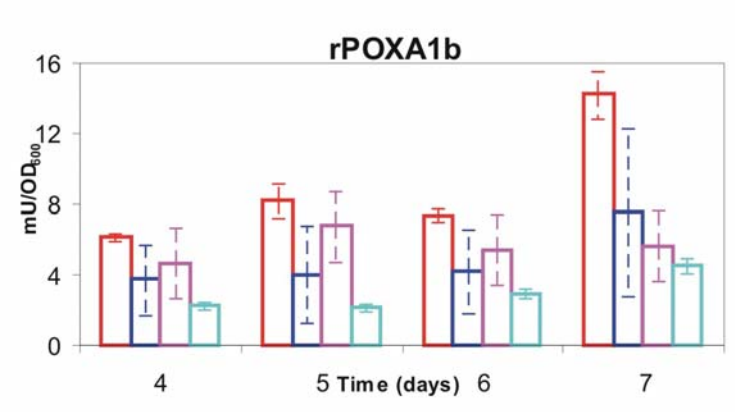

Fig. 2.18 Specific production of rPOXA1b and rPOXC. Nutrient effect on laccase secretion. Cultures were grown in selective medium supplemented with 0.6mM $CuSO_4$ □, SD (selective medium containing 20 g l^{-1} glucose); □, Sd (selective medium containing 10 g l^{-1} glucose); □, SG (selective medium containing 20 g l^{-1} galactose); □, Sg (selective medium containing 10 g l^{-1} galactose).

2.2.2 rPOXA1b purification and characterisation

The recombinant laccase was purified by ultrafiltration and cation exchange chromatography from which it eluted at 0.28 M NaCl (Fig. 2.19). Purification of the recombinant protein is summarized in table 2.13.

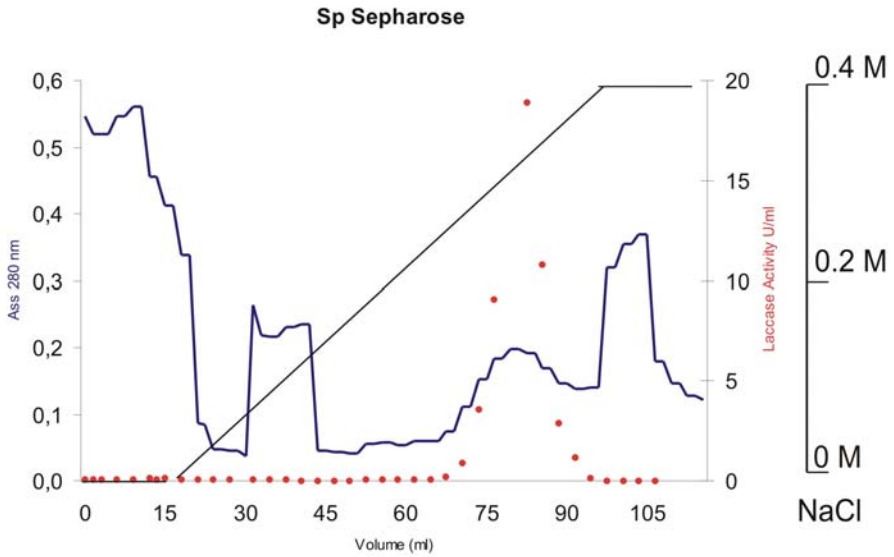

Fig. 2.19 Elution profile of rPOXA1b.

Table 2.13 rPOXA1b purification from *Saccharomyces cerevisiae* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	198	94	2.1	100	--
Ultra-filtrated broth	124	16.3	7.6	62.6	3.6
S Sepharose	86.7	3.2	27.1	43.8	12.9

Comparison between SDS-PAGE (Fig. 2.20A) and Western blot analysis (Fig. 2.20B), allowed the identification of the recombinant protein as that one exhibiting an apparent molecular weight of 131kDa. SDS-PAGE analysis showed that the relative abundance of the protein of interest after cationic exchange chromatography step was very low. When analysed by native PAGE recombinant protein displayed a lower mobility than the native one (Fig. 2.20C). On IEF the rPOXA1b exhibited a more acidic pI than POXA1b (Fig. 2.20D).

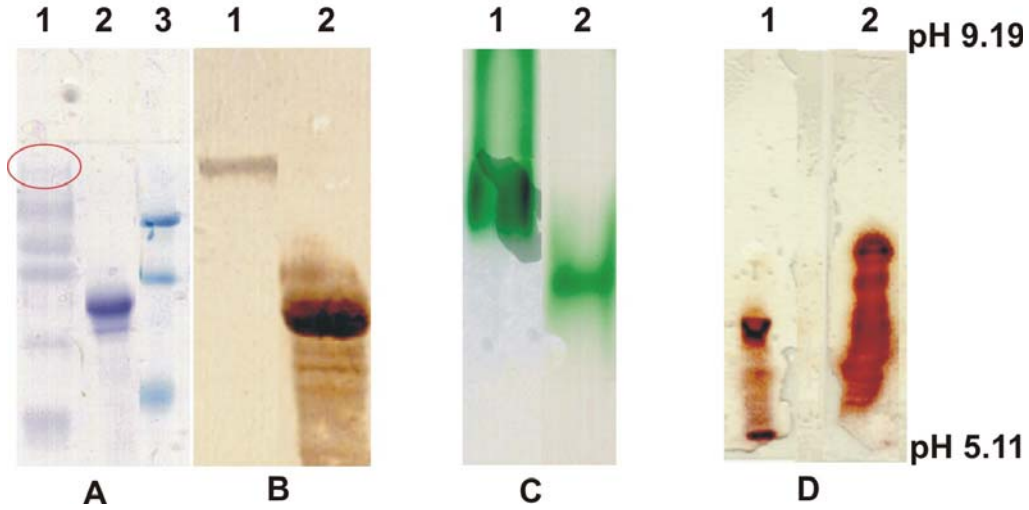

Fig. 2.20 Electrophoretyc analyses of rPOXA1b. Lane 1 recombinant protein; lane 2 native protein; lane 3 molecular weight standards (116 kDa, 66.2 kDa, 45 kDa, 35 kDa). (A) SDS-PAGE, Comassie staining; (B) Western blotting; (C) NATIVE PAGE, ABTS staining; (D) IEF, 2,6-DMP staining.

Only affinity constants of the recombinant protein (Table 2.14) were determined because catalytic constants cannot be calculated for unpurified protein. No marked differences between native and recombinant POXA1b were observed with 2,6-DMP. On the other hand the recombinant protein has a four fold higher affinity towards ABTS than that of the native protein.

Table 2.14 rPOXA1b affinity constants compared with those of native POXA1b.

Substrate	K_M(mM)	
	POXA1b	r-POXA1b
ABTS	(0.47 ± 0.06)	(0.119 ± 0.004)
2,6-DMP	(0.26 ± 0.09)	(0.298 ± 0.030)

2.3 P. ostreatus *laccases tailoring*

The *P. ostreatus* laccase expression systems developed in *S. cerevisiae* was used as tool to start directed evolution experiments.

2.3.1 Randomly mutated cDNAs libraries construction

The mutated cDNAs libraries were created randomly mutating the cDNAs at a fixed mutagenic rate. The mutation frequency of an amplification reaction is determined by the formula:

$$\text{mutation frequency} = \text{error rate} \times d$$

where *mutation frequency* is expressed as mutations/kb, *error rate* is the error rate of the DNA polymerase in errors/(kb x duplication), and *d* is the number of duplications during PCR experiments.

One to two amino acid changes per gene per generation was chosen as rate to perform the first approach of random mutagenesis on laccase cDNAs. Figure 2.21 is a simplification of the mutagenesis mechanism.

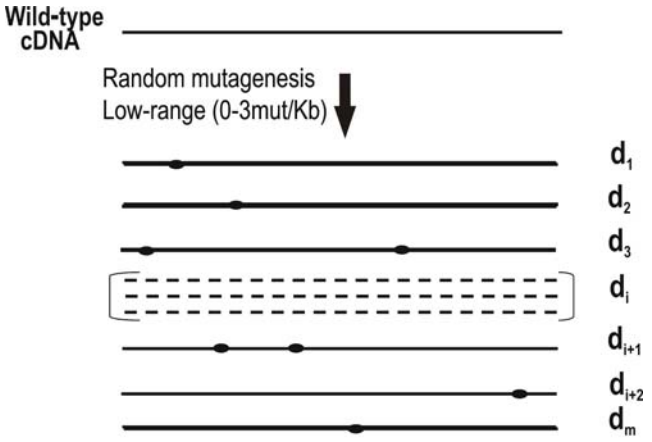

Fig 2.21 Random prone PCR.

This rate was chosen as random mutagenesis at low error rates favours the discovery of beneficial mutations at structurally tolerant sites, which are more abundant on the surface and in loop regions of a protein. Changes in the closely packed interior and within secondary structural elements (likely to arose at a high frequency of mutation) are more likely to be disruptive (Voigt *et al.*, 2001). Libraries of mutants have to be made complex enough to contain rare beneficial mutations. Therefore, several iteration of the low rate mutational PCR were used to increase the number of mutations.

2.3.2 Homologous recombination

Expression of mutated laccase library in *S. cerevisiae* was performed taking advantage of the high level of homologous recombination in yeast, which makes *in vivo* approaches fast, efficient, and non-mutagenic. The optimal length of the homologous overhangs is usually over 100 bp on both 5' and 3' –ends. Overhangs can be generated by restriction of the cDNAs on the basis of the restriction map of the recombinants vectors. This study was performed starting from the most promising construct for both cDNAs, C-pSAL4 and

B-pSAL4. Appropriate enzymes must cut the cDNA once or twice (if a deletion is wanted) in the latter case leaving at least 40 bp on each edge, and must not cut into the plasmid. Restriction maps of *poxa1b* (Fig. 2.22A) and *poxc* (Fig. 2.22B) are shown in the figure 2.22.

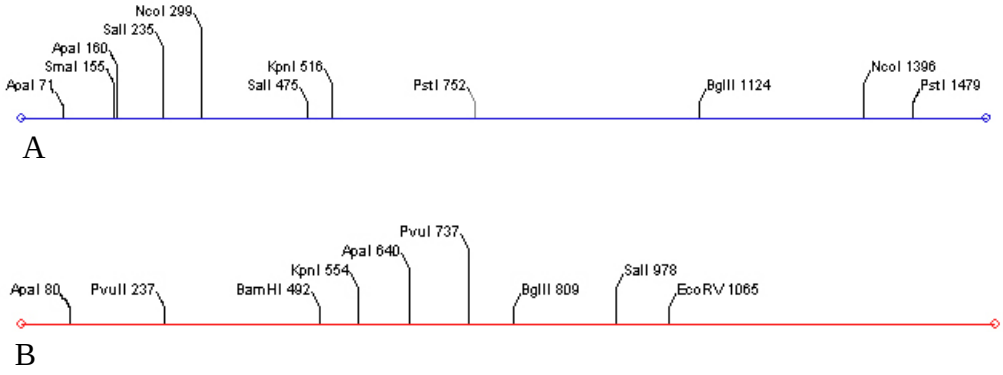

Fig. 2.22 Restriction maps of laccase cDNAs. (A) *poxa1b*; (B) *poxc*.

From a comparison of these maps with the plasmid restriction map, and considering the much lower activity of rPOXC than that of rPOXA1b, *poxa1b* cDNA was chosen as the target sequence for the homologous recombination. The B-pSAL4 construct was then cut with SmaI and BglII restriction enzymes leaving 155 bp at 5'-end and 475 bp at 3'-end of the original *poxa1b* cDNA sequence (Fig. 2.23).

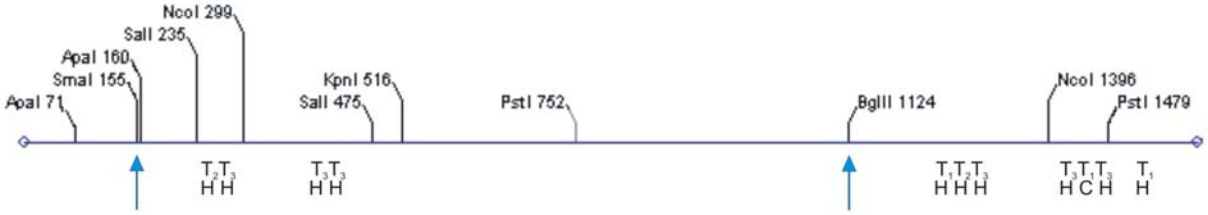

Fig. 2.23 Cutting site of *poxa1b* cDNA.

Figure 2.23 highlights the cutting site and shows the position of copper coordination sites. The overhangs will allow homologous recombination between the plasmid and the mutated cDNAs within the yeast cells allowing the closed plasmid to be maintained and propagated again.

Mutant *poxa1b* inserts were cotransformed with open plasmid that contains sequences homologous to the ends of the poxa1b cDNAs on both ends. Homologous recombination combines these to form complete plasmids. Negative control was performed transforming the yeast cells only with the B-pSAL4 open plasmid carrying poxa1b cDNA termini. Yeast cells, where homologous recombination between B-pSAL4

plasmid and mutated cDNAs takes place (Fig. 2.24), will be able to grow on selective medium, since they carry out a closed plasmid.

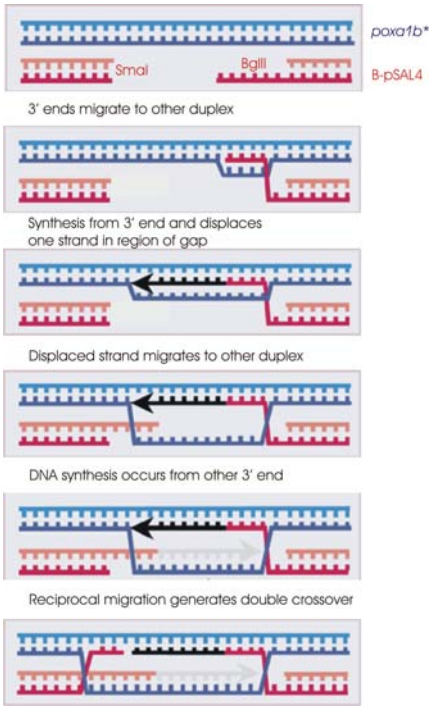

Fig. 2.24 Scheme of the mechanism of homologous recombination in transformed yeasts.

After transformation, yeast cells were spread on selective medium supplemented with guaiacol for laccase activity plate test. No clone was present on control plates, whilst recombination plate exhibited tens of clones displaying different level of activity. More than one hundred transformants were obtained after the first round of random mutagenesis and homologous recombination experiments. A selective plate, on which several transformants were grided is shown on figure 2.25. On this plate a red circle highlights yeast cells secreting wild type POXA1b, brown halo indicates the oxidation of the substrate by secreted laccase.

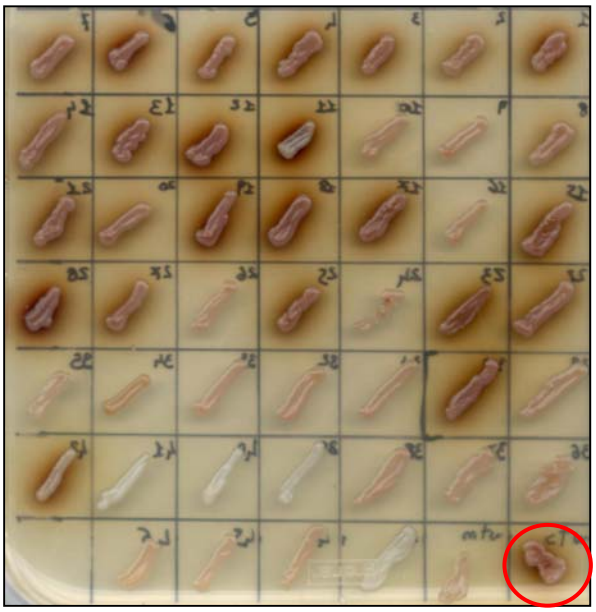

Fig. 2.25 Yeast cells secreting randomly mutated rPOXA1b. Wild type rPOXA1b is indicated by a red circle.

2.3.3 Transformants analysis

The third step of tailoring experiments was the development of a rapid screening method to identify those mutants with optimal combination of beneficial mutations.

The screening procedures employed in approaches based on random mutagenesis must be both suitable for high throughput analysis and sufficiently discriminative. Moreover, screening procedures must allow the identification of the desired mutant using a quick, easy, reproducible and inexpensive assay. Since it is possible that a single mutant does not display the optimal characteristics all together, the assay conditions have to take into account a single variable at a time (i.e. temperature, pH, stability, specificity), allowing, anyway, their combinations.

The method used discriminates between the growth rate and the mutant characteristic. Yeasts cells were counted under a microscope to normalize their growth on plate. A PVDF membrane and a carbonate filter were placed on the solid medium, and cells were deposited on the latter filter. The porosity of the filters allowed the cells to assume nutrients from the medium while the secreted protein were entrapped in the PVDF membrane. The PVDF membrane with immobilised secreted proteins was then used for laccase activity tests. The colonies lifted carbonate membrane was used on buffer-substrate plates (at different pHs) and/or incubated at different temperatures: the rate of development of a coloured halo, and/or the intensity and the diameter of the halo were used as indicator of the relative performances of the mutants.

Selected clones can be further mutated and/or randomly recombined for the next round of expression and selection.

3 DISCUSSION

The first heterologous expression of functional laccase was described at the beginning of the nineties (Kojima *et al.*, 1990). From then, although many of laccase genes have been cloned and sequenced from several fungi, only few laccases have been successfully expressed in heterologous yeast hosts. This scarcity of examples can be linked to the low expression capacity of yeasts as compared to filamentous fungi systems, but also to the fact that these microorganisms are not able to process different laccase enzymes post-translationally with good efficiency (Klonowska *et al.*, 2004).

In this work, laccase cDNAs *poxc* and *poxa1b* from *P. ostreatus* have been used to develop expression systems in the two yeasts *K. lactis* and *S. cerevisiae*, and preliminary directed evolution experiments have been performed.

Laccase expression in yeasts has been performed using both native laccase or yeast derived signal peptides (*S. cerevisiae* α-factor, invertase) and different results were reported for various expressed enzymes. In *P. pastoris* better results have been obtained with the laccase leader peptide as compared to *S. cerevisiae* α–factor fusion (Jönsonn *et al.*, 1997; Liu *et al.*, 2003, Soden *et al.*, 2002), whereas other authors have found comparable extracellular activities via the two signal peptides (Otterbein *et al.*, 2000). In *S. cerevisiae*, a higher secreted activity has been reported for fusion with the yeast α-factor leader peptide (Kiiskinen and Saloheimo, 2004) or with the yeast invertase leader peptide (Klonowska *et al.*, 2004). Therefore, since no univocal results have been obtained, the best performing leader peptide to drive the secretion of recombinant laccases in yeasts is apparently not predictable.

Considering that the control of protein translocation can be the bottleneck in many cases, the effect of both the endogenous laccase signal peptide and the killer toxin leader sequence on the recombinant expression of POXC in *K. lactis* have been examined. Intra and extra-cellular laccase production were analysed. No production of the recombinant enzyme in both compartments was detected by immunoblot analysis when the killer toxin leader sequence was used. On the other hand, laccase signal peptide allowed the production and the secretion of an active rPOXC in the medium. These data indicates that the leader peptide not only affects the secretion, but also the production of recombinant enzyme. On the basis of this result POXA1b recombinant expression was only performed using its native signal peptide.

For the heterologous expression of *P. ostreatus* laccases in *S. cerevisiae* three expression vectors were constructed for each protein, differing from each other for replication origins, promoter regions and secretion leader sequences.

Replication origin: the presence of a 2µ sequence, which allow to maintain the expression plasmid at a high copy number in the cell, correlated with a high production of secreted activities relative to that observed with CEN/ARS containing constructions present in monocopy in the cell (see B-pAC1 *versus* B-pSAL4 and B-Yep). Such a result can be expected every time the expressed protein does not interfere with cell growth.

Signal peptide: considering vectors with the same origin of replication, laccase leader peptides were found to be more effective to drive laccase secretion than the yeast signal peptide I used (*S. cerevisiae* invertase) (see B-Yep *versus* B-pSAL4).

In the inferred conclusions, the influence of the promoter is not considered as the promoters used are either constitutive (*ADHI* and *PGK*) or inducible (*CUP1*) but used as a constitutive promoter since copper, the inductor, was always present in the culture as laccase cofactor. The construction containing a 2µ as replication origin, a homologous laccase signal peptide, and a copper inducible *CUP1* promoter region, gave the best results for both laccases.

Hence, the native signal peptide seems to be more efficient in producing extracellular *P. ostreatus* laccases, either in *K. lactis* or *S. cerevisiae*.

It has to be noted that one nucleotide in *poxa1b* cDNA used for the expression deviates from the known genomic sequence: this substitution, found in all the clones sequenced, exchanges an N for D in position 201, and removes one of the six potential N-glycosylation sites. The amplified cDNA may be a natural allelic variant, since the *P. ostreatus* strain used was a dikaryon.

Western blotting analyses of cellular extracts and culture supernatant of the yeast strains producing *P. ostreatus* laccases showed that the produced recombinant proteins are almost equally distributed between the two *K. lactis* cellular compartments, whilst the majority of laccase produced by *S. cerevisiae* is located in yeast cells. Moreover, the apparent molecular mass of secreted rPOXA1b is slightly higher in *K. lactis* (11.3% by SDS-PAGE), and significantly higher (69% by SDS-PAGE) in *S. cerevisiae* than that of the native protein. The protein recognised by the anti-POXC antibodies has a Mr very closed to that of the native laccase for both hosts and in both cellular compartments (1.6% difference by SDS-PAGE). These data showed that yeasts differently glycosylate the proteins, confirming the low tendency of *K. lactis* respect to *S. cerevisiae* to hyperglycosylate recombinant proteins.

Levels of laccase secreted activity were markedly different: rPOXA1b transformants always gave much higher activity than rPOXC transformants, and production of both laccases in *S. cerevisiae* was significantly lower than that in *K. lactis*. These results were obtained for all the *S. cerevisiae* vectors in all the growth conditions tested (i. e. inducer concentration, medium composition, copper concentration).

Based on the production kinetics and western blotting analyses of yeasts strains producing *P. ostreatus* laccases, the factor limiting production appears to include inefficient secretion. Since laccase production was conducted in both hosts in the same conditions, a lesser efficient secretion may explain the lower level of secreted laccase activities in *S. cerevisiae* compared to *K. lactis.* On the other hand, rPOXC transformants activity is always significantly lower than that of rPOXA1b transformants, therefore it seems to be affected by some factors other than inefficient secretion. A low stability could explain the inefficient rPOXC production, as a fact Bulter *et al.*, (2003) drastically improved the heterologous expression of *M thermophila* laccase in *S. cerevisiae* by directed evolution experiments, and hypothesised that improved stability allowed more enzyme to be secreted.

Since better performances for the production of *P. ostreatus* laccases were obtained with *K. lactis* as heterologous laccase producer, further characterizations were carried out in this expression system.

Recombinant laccases were purified to homogeneity, characterized, and their properties compared to those of the corresponding native proteins. Amounts of the purified rPOXA1b and rPOXC were similar (1.1 and 1.4 mg l^{-1}, respectively, taking into account the purification yield). It is worth to note that rPOXC specific activity is about two orders of magnitude lower than that of the native one, whereas no significant difference was observed between rPOXA1b and native POXA1b specific activities. When the effect of copper concentration on *K. lactis* laccase production was evaluated, rPOXC showed a positive correlation between copper availability and laccase activity up to 0.5 mM $CuSO_4$, higher $CuSO_4$ concentration had no further effect. Moreover, no variation of activity was observed when purified rPOXC was incubated with either Cu(II) or Cu(I), to the contrary of what Larrondo *et al.* (2003) observed with a *Ceriporiopsis* laccase. These results lead to hypothesize that rPOXC could undergo an incomplete or incorrect folding not strictly dependent on copper availability.

The recombinant enzymes show higher molecular weights than their native counterparts with a more marked difference in the case of POXA1b (11.3% by SDS-PAGE) than POXC (1.6% by SDS-PAGE). Mass spectrometry analyses allowed to validate the primary structures of rPOXA1b and rPOXC, and to determine their glycosydic moiety structures. POXC contains two potential N-glycosylation consensus sites in its sequence, but only one of them being modified both in the recombinant and in the native protein. The oligosaccharide chains linked to the pentasaccharide core ranged from three to seven mannose residues for N444 in the recombinant protein, and native protein shows the same site with up to six mannose residues linked to the pentasaccharide core (Giardina et al, 1996). POXA1b contains more potential N-glycosylation consensus sites in its sequence relative to POXC, and three of them were found modified in the recombinant protein as well as in the native one. The oligosaccharide chains linked to the pentasaccharide core ranged from five to eight mannose residues for N294, from one to ten mannose residues for N434, and from zero to four mannose residues for N470. No information was found regarding the residue N342, whilst N490 was found not modified. Native protein shows N294, N470 and N490 with up to five mannose residues linked to the pentasaccharide core, N434 not modified, whilst no information was available regarding residue N342 (Giardina et al, 1999). Consequently, the extent of glycosylation per site should affect the molecular weight of the heterologously expressed enzymes more than the number of N-glycosylated sites itself.

It is worth to note that rPOXA1b undergoes a C-terminal processing, as well as the *P. ostreatus* native protein (Giardina et al, 1999). Indeed, rPOXA1b was found depleted of the last two amino acids (mapped C-terminal sequence: …LPAPL). Three overlapping C-terminal sequences with different relative abundance were found for native POXA1b by automatic C-terminal sequence analysis (Giardina et al, 1999): …LPAPLK (40-45%); …LPAPL (40-45%); and …LPAP (10-20%).

Comparing the kinetic parameters determined for the recombinant POXA1b laccases obtained both from *K. lactis* and *S. cerevisiae* with those of the native *P. ostreatus* POXA1b, it is possible to note that both the recombinant enzymes exhibit a higher affinity for ABTS. The main structural differences found between native and recombinant POXA1b are extent of glycosylation and C-terminal sequences. Considering that *K.*

lactis rPOXC, whose extent of glycosylation is similar to that of the native one, also exhibited a higher affinity for ABTS, different extent of glycosylation should not affect the affinity towards this non-phenolic substrate.

On the other hand, a role of the C-terminus in modulating enzymatic activity has been hypothesised, at least for ascomycete laccases (Hakulinen *et al.*, 2002; Kiiskinen and Saloheimo, 2004). A unique C-terminus for *K. lactis* rPOXA1b was determined, whilst the native protein exhibited three differently abundant C-terminal sequences. If C-terminus can really affect laccase activity, the C-terminus differences found between the native and recombinant POXA1b could be related to their different affinity towards ABTS. The lack of information regarding the C-termini of the other recombinant laccases does not allow inferring general conclusions. Further investigations are needed to verify and to evaluate functional role of the C-terminal processing.

Performances of the *K. lactis* expression system (efficient secretion ability, weaker tendency to hypermannosilate proteins, and ease of manipulations) make this yeast a better producer than *S. cerevisiae*, at least for *P. ostreatus* laccases, and a promising host for deeper investigations. Nevertheless, *S. cerevisiae* is the yeast that reached the forefront in experimental molecular biology, being the first eukaryotic organism whose entire genome sequence has been made available (Goffeau *et al.*, 1996; Dujon, 1996). No wonder then that the wealth of sequence information, the great detailed biochemical studies, the high recombination frequency, added to the ease of manipulation made this yeast the most commonly used host organism in directed evolution experiments. For this reason, the results obtained with recombinant expression of *P. ostreatus* laccases in *S. cerevisiae* encouraged me to project new oxidative catalysts using molecular evolution techniques. Therefore, the *S. cerevisiae* laccase expression systems were further used to set up directed evolution experiments, despite the fact that the amount of secreted laccase was not very high. Mutated cDNAs libraries with different mutation rate were created, and homologous recombination experiments were performed, giving rise to libraries of mutated laccase secreting yeasts. Moreover a screening procedure to select for clones exhibiting desired property was realized.

This thesis work allowed obtaining the heterologous expression of two *P. ostreatus* laccases in two different yeast systems as well as to purify and characterise the recombinant proteins. Moreover, this research work broadened the potentiality of the developed expression system to the tailoring the enzymes in order to address them to such large markets as pulp and textile bleaching, and enzymatic remediation of waste streams.

A new laccase host (*K. lactis*) has been built on, and its promising performances will lead to further investigate its utilization for further structure-activities studies, as well as for directed evolution. Results obtained demonstrate the potential of the recombinant expression for the study of enzymes of potential industrial interest.

4 APPENDIX

4.1 *Introduction*

C30 is a white-rot basidiomycete that colonizes the evergreen oak (*Quercus ilex* L.) leaf litter in the Mediterranean area (Tagger *et al.*, 1998). This particular isolate has previously been grouped with strains of *Marasmius quercophilus* (Tagger *et al.*, 1998), but recent phenotypic and molecular evidence have suggested that it belongs to the *Coriolaceae* (and more precisely to the *Trametes* genus (Klonowska *et al.*, 2003)), rather than the *Tricholomatacea*. Considering the biotope from which *C30* was isolated, delignification is essential to this fungus, and laccases may be key enzymes involved in this process (Tagger *et al.*, 1998). Evidences that this fungus is able to produce at least 4 isoforms have been obtained (Klonowska *et al.*, 2001). A constitutive, LAC1, as well as an inducible enzyme, LAC2, were purified and characterized and the corresponding genes were sequenced (Dedeyan B. *et al.*, 2000; Klonowska *et al.*, 2002). LAC1 is a high redox potential enzyme (E^0 = 0.73 V) with moderate activity, whereas LAC2 is a low redox potential enzyme (E^0 = 0.56 V) with unusually high oxidative capacities. In addition, a third laccase encoding sequences, *lac3*, was recently cloned from *Trametes* sp. strain C3O and expressed in *S. cerevisiae* (Klonowska *et al.*, 2004). The data on the recombinant laccase show that, like LAC1 and LAC2, LAC3 is an acidic enzyme, and belongs to the "low" redox potential group of laccases (E^0 = 0.53 V). Despite its low redox potential, when compared to laccases purified from other fungi, LAC3 appears to be a remarkably efficient enzyme. In fact it is as efficient as the most efficient high redox potential laccases like *P. ostreatus* and *T. trogii* enzymes (Garzillo *et al.*, 2001) at least on SGZ and ABTS (Klonowska *et al.*, 2004). On the high redox potential phenol substrate GUA, LAC3 catalytic efficiency is apparently 2 to 3 orders of magnitude higher than the high redox potential *Polyporus pinsitus*, and the low redox potential *Scytalidium thermophilum* and *Myceliophthora thermophila* (Klonowska *et al.*, 2004). LAC3 activities on phenolic compounds seem only partly related to its specific T1 copper redox capacities. As phenolic oxidation involves a proton release, factors like hydrogen bonding or the extend of protonation of ionizable groups in the vicinity of the T1 copper probably have considerable effects on the overall catalytic efficiency. Fig. 4.1 shows the superposition of models of C30 enzymes (T. Tron, personal communication) on the structure of *C. cinereus* laccase (Ducros *et al.*, 1998). In these models, polypeptide chain length variations of 1 to 3 residues in the loop containing the T1 copper proximal ligand induce structural changes around the copper. Such differences in the main chain conformation around the copper might play a role in tuning the laccase potential and/or controlling the substrates accessibility.

With the aim to deeply understand the role of the chain conformation around the copper, site directed mutagenesis experiments were performed (A. Klonowska, T. Tron, personal communication). LAC3 mutants were constructed changing the minimum number of amino acids in the loop containing the proximal T1 coordinating histidine to make LAC3 resemble LAC1: in rLAC3^m ΔN390 N 390 was deleted, and in rLAC3^m A397$\rightarrow$F the A in position 397 was exchanged with a F.

Fig. 4.1 3D models of T1 copper pocket region from C30 laccases. The structures have been computerized with Modeler and Procheck softwares using *C. cinereus* laccase data as template (Ducros *et al.*, 1996).

4.2 *Materials and methods*

Enzyme production.

Yeasts were cultivated at 28°C. Precultures were obtained in two stages from a single colony freshly grown on a selective plate. Cells were first grown in 15 ml tubes containing 5 ml of SD Medium for 48 h on a rotating wheel. A volume of suspension sufficient to reach a final OD_{600} value of 0.05 was then used to inoculate 250 ml Erlenmeyer flasks containing 50 ml of SD and cells were then grown for 48 h on a reciprocal shaker. Bio-reactor cultivations (batch) were performed in a 3 l fermentor vessel (B. Braun, Biotech International GmbH) containing 2 L of SD. The inoculum was added to a final OD_{600} value of 0.05 and yeasts were grown under stirring (250 rpm) and an air flow of 16 L/H. Samples (1 ml) used for laccase activity and cell density were withdrawn and analysed regularly throughout cultivation.

Laccase activity.

Routine laccase assay was based on syringaldazine (SGZ) oxidation in 0.1 M phosphate buffer (pH 5.7) at 30°C. Oxidation of SGZ was monitored spectroscopically by absorbance measurements at 525 (ε = 6.5 10^4 M^{-1} cm^{-1}).

Recombinant LAC3 purification.

Cells were sedimented by centrifugation at 1,600 x g and 4°C for 10 min. Culture supernatant (2 l) was mixed with succinate buffer (100 mM, pH 5.5) pre-equilibrated DE-52 (Whatman) slurry and filtered on a n°3 fritted glass to eliminate dark brown matter that sticks to resin. Filtrate was concentrated 10 fold by ultrafiltration using YM10 membranes (Amicon, Millipore, Bedford, MA, USA), buffer exchanged for 20 mM phosphate, pH 5.7 (buffer A) and finally applied to an ion-exchange DEAE-Sepharose column (2.5 x 20 cm, Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) equilibrated with the same buffer. Proteins were eluted at a flow rate of 4 ml min^{-1} with a step gradient of NaCl: 0.1 M, 0.15 M, 0.2 M, 0.25 M, 0.3 M, 0.35M, 0.4M, and 0.5M each for 25 minutes. Enzyme purity in active fractions was then confirmed by SDS-PAGE.

Enzyme characterization.

Determination of protein concentration, SGZ oxidation tests, native and denaturating PAGE were as previously described

4.3 *Results and discussion*

rLAC3

When the rLAC3 secreting yeast was grown in a 2 l fermentor culture, total laccase activity reached a maximum value of 0.4 U ml^{-1} within four days (data not shown). Subsequently, the culture broth was concentrated by ultrafiltration. The active sample was separated by anionic exchange (Fig. 4.2) chromatography and eluted with a step gradient.

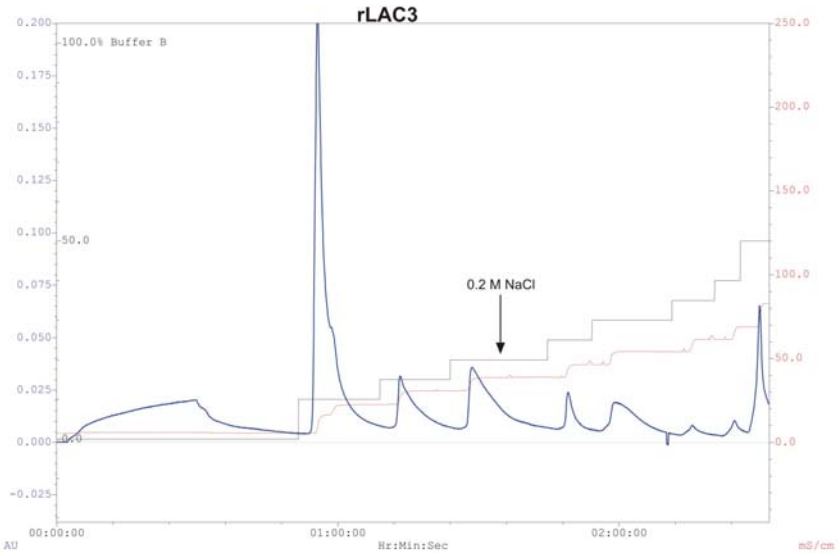

Fig. 4.2 rLAC3 elution profile from the anion exchange chromatography.

Fractions of the gradient were assayed for laccase activity: most of the laccase activity eluted at 0.2 M NaCl and was found homogeneous by SDS-PAGE (Fig. 4.5). rLAC3 was obtained with a specific activity of 302.90 U/mg, for a final yield of 71.45% (Table 4.1).

Table 4.1 rLAC3 purification from *Saccharomyces cerevisiae* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	585	28.86	20.27	100	--
Ultrafiltration	618	6.09	101.48	105	5.0
DEAE-Sepharose	418	1.38	302.90	71.45	14.9

rLAC3^{m} ΔN390

When the rLAC3^{m} ΔN390 secreting yeast was grown in a 2 l fermentor culture, total laccase activity reached a maximum value of 0.21 U ml^{-1} within three days (data not shown). Subsequently, the culture broth was purified from dark brown matter with a batch chromatography followed by ultrafiltration. The active sample was then separated by anionic exchange chromatography (Fig. 4.3) and eluted with a step gradient.

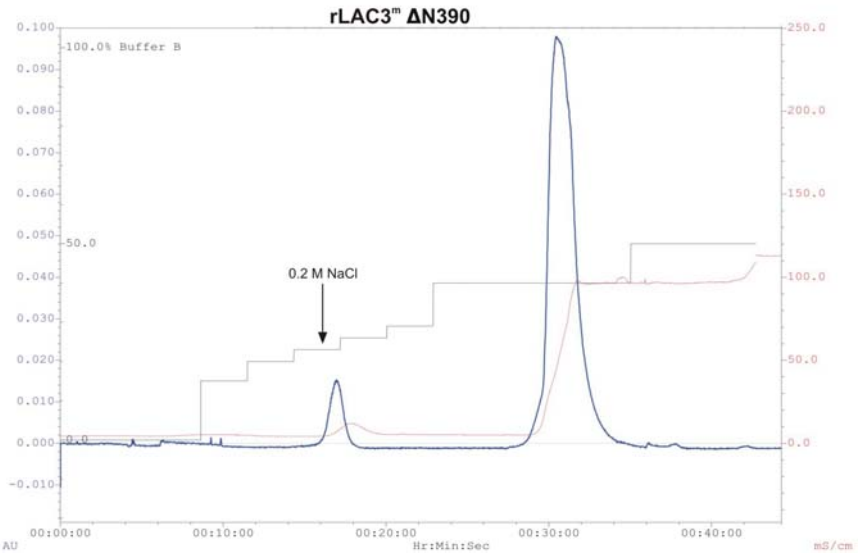

Fig. 4.3 rLAC3^{m} ΔN390 elution profile from the anion exchange chromatography.

Fractions of the gradient were assayed for laccase activity: most of the laccase activity eluted at 0.2 M NaCl and was found homogeneous by SDS-PAGE (Fig. 4.5). rLAC3^{m} ΔN390 was obtained with a specific activity of 333.3 U/mg, for a final yield of 21.9% (Table 4.2).

Table 4.2 rLAC3^{m} ΔN390 purification from *Saccharomyces cerevisiae* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	456	54	8.4	100	--
Batch-DEAE	148	1.71	86.5	32.4	10.3
DEAE-Sepharose	100	0.3	333.3	21.9	39.7

rLAC3^{m} A397→F

When the rLAC3^{m} A397→F secreting yeast was grown in a 2 l fermentor culture, total laccase activity reached a maximum value of 0.22 U ml^{-1} within three days (data not shown). Subsequently, the culture broth was purified from dark brown matter with a

batch chromatography followed by ultrafiltration. The active sample was then separated by anionic exchange (Fig. 4.4) chromatography and eluted with a step gradient.

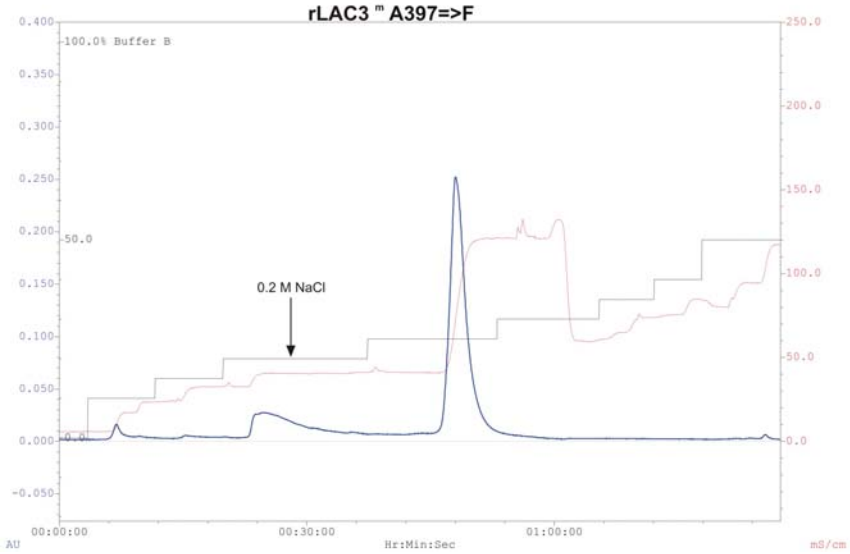

Fig. 4.4 rLAC3 ^{m} A397→F elution profile from the anion exchange chromatography.

Fractions of the gradient were assayed for laccase activity: most of the laccase activity eluted at 0.2 M NaCl and was found homogeneous by SDS-PAGE (Fig. 4.5). rLAC3^{m} A397→F was obtained with a specific activity of 384.9 U/mg, for a final yield of 77.6% (Table 4.3).

Specific activities of the three recombinant laccases (the original rLAC3 and two mutants) were not found significatively different. When the recombinant LAC3 and its mutant forms where analysed by SDS-PAGE (Fig. 4.5), rLAC3^{m} ΔN390 exhibited a slightly lower apparent molecular mass, whilst rLAC3^{m} A397→F apparent molecular mass was similar to that of wild-type rLAC3.

Table 4.3 rLAC3^{m} A397→F purification from *Saccharomyces cerevisiae* culture.

Purification Step	Total activity	Total protein	Specific activity	Recovery	Purification Fold
	U	mg	U/mg	%	
Broth	362	30.77	11.76	100	--
Batch-DEAE	310	1.58	196.0	86.1	16.6
DEAE-Sepharose	281	0.73	384.9	77.6	32.7

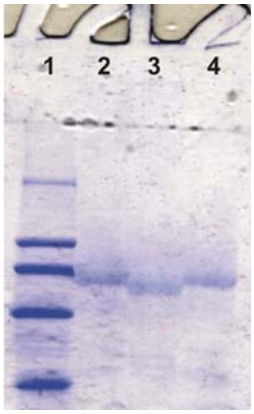

Fig. 4.5 Electrophoretyc analysis of recombinant LAC3 isoenzymes (2 µg). Lane 1 molecular weight standards (200 kDa 116.25 kDa, 97.4 kDa, 66.2 kDa, 45 kDa); lane 2 rLAC3; lane 3 rLAC3^{m} ΔN390; lane 4 rLAC3^{m} A397→F.

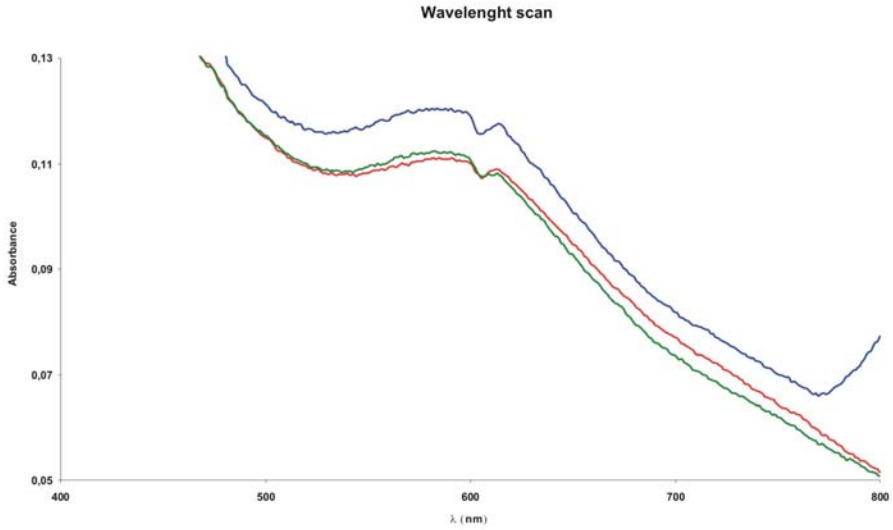

Fig. 4.6 Wavelength scan spectrum of recombinant LAC3. — rLAC3; — rLAC3^{m} ΔN390; — rLAC3^{m} A397→F.

Wavelength scan spectra of the three recombinant laccases were recorded (Fig. 4.6). In our experimental conditions, the nature of the absorption band at 600nm did not reveal significant differences between the wild type and the two mutants LAC3.

The EPR spectra, recorded in the same conditions for the 3 proteins did not reveal any differences either (V. Belle, T. Tron, personal communication). Therefore, at this stage of the study on rLAC3 and two of its mutants, it seems that the neither ΔN390 or A397→F mutations affect significantly the spectral properties or the specific activity of the enzyme. Our production of recombinant LAC3, both as native and mutated forms will be a basis for a study to better understand the role of the peptide chain conformation around the copper in particular through the measurement of their T1 copper redox potential.

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6 COMMUNICATION - PUBLICATION

"Sviluppo di sistemi per l'espressione eterologa in procarioti della Fenolo-ossidasi POXC da *Pleurotus ostreatus*". (V. Rivieccio, A. Piscitelli, P. Giardina, M. L. Tutino, and G. Sannia). Riunione interregionale SIB 28-29 Giugno 2001, Salerno.

"Recombinant expression of *Pleurotus ostreatus* laccases in yeasts: a basis for directed evolution". (A. Piscitelli, V. Faraco, P. Giardina, T. Tron, and G. Sannia). Oxizymes in Naples 3-5 June 2004

"Laccases: natural sources and molecular evolution, two routes to optimized catalysts". (C. Gaudin, G. Iacazio, A. Piscitelli, and T. Tron). Oxizymes in Naples 3-5 June 2004

"Recombinant expression of *Pleurotus ostreatus* laccases in *Kluyveromyces lactis* and *Saccharomyces cerevisiae*". (A. Piscitelli, P. Giardina, C. Mazzoni, and G. Sannia). *Appl. Environ. Microbiol.* 2004, Submitted for publication

RECOMBINANT EXPRESSION OF *PLEUROTUS OSTREATUS* LACCASES IN *KLUYVEROMYCES LACTIS* AND *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding different isoenzymes. Among these, POXC is the most abundantly produced, and POXA1b the most stable at alkaline pH. The corresponding cDNAs were expressed for the first time in the heterologous hosts *Kluyveromyces lactis* and *Saccharomyces cerevisiae*. Vectors containing *P. ostreatus* laccase cDNAs with their own or yeast signal peptides (*K. lactis* killer toxin or *S. cerevisiae* invertase) were constructed and used to transform both hosts. Secretion in the extracellular medium of rPOXC and rPOXA1b as active proteins was obtained, albeit *K. lactis* was much more efficient than *S. cerevisiae*. Parameters affecting production of recombinant proteins were optimised, rPOXA1b transformants showing always higher secreted activity than rPOXC transformants.

The recombinant laccases from *K. lactis* were purified to electrophoretic homogeneity and characterized. rPOXA1b specific activity was similar to that of the native protein, whilst rPOXC specific activity was much lower than that of the native POXC. An incomplete or incorrect rPOXC folding was hypothesised. Mass spectrometry analyses of the recombinant proteins allowed to verify their primary structures and to identify post-translational modifications. Both recombinant laccase signal peptides were correctly cleaved, and rPOXA1b was found depleted of the last two amino acids. Data reported confirmed *K. lactis* as an efficient recombinant protein producer, with a lower tendency to hyperglycosylate recombinant proteins respect to *S. cerevisiae*, and proved it as a suitable system for fungal laccase production.

INTRODUCTION

Laccases (E.C.1.10.3.2, *p*-benzenediol:oxygen oxidoreductase) belong to the blue oxidase family of proteins, along with ascorbic oxidases and ceruloplasmins. These copper-containing enzymes (multicopper oxidases) couple the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water (41). Laccases are widely distributed in higher plants and fungi, and presence of laccase or laccase-like activity has also been found in some insects (7, 23) and bacteria (6). Roles attributed to these enzymes include pigmentation in fungi and bacteria (1, 40), lignification and delignification of plant cell walls (29), virulence factors (40), and copper and iron homeostasis (39). Laccases contain four copper atoms, central to the enzyme catalytic mechanisms (41), distributed between a mononuclear site (one type-1 copper) and a trinuclear site (one type-2 and two type-3 copper).

Laccases have a very broad substrate range, being able not only to oxidize polyphenols but also aminophenols, methoxyphenols, and aryl amines. Consequently, laccases are increasingly investigated for a variety of practical applications ranging from their use in the pulp and paper industry, in bioremediation, and for analytical purposes (27).

The white-rot fungus *Pleurotus ostreatus* is able to express multiple laccase genes encoding isoenzymes with different physico-chemical and kinetic characteristics; moreover the amount of the specific enzymes produced depends on culture conditions. So far, five isoenzymes secreted by the mycelium have been purified and characterized: POXC (31), POXA1w (32), POXA1b (12), and, more recently, the two strictly related isoenzymes POXA3a and POXA3b (33). POXC is the most abundantly produced in all growth conditions tested so far; POXA1w shows peculiar differences with regard to metal ions content; POXA1b is the most stable at alkaline pH; POXA3a and POXA3b are heterodimeric laccases. Studies on the laccases encoding genes have also led to the identification of four different genes and of the corresponding cDNAs, *poxc* (previously named *pox2*) (11), *pox1* (which codes for a laccase isoenzyme not identified yet) (10), *poxa1b* and *poxa3* (12, 33).

Biotechnological and environmental applications require large amounts of readily available crude and/or purified enzymes. Laccases secreted from wild-type fungal organisms may not be suitable for commercial purposes as low enzyme yields, and undesirable preparation procedures can not be economically advantageous. Therefore, it is thought that heterologous expression of laccases in different hosts could be a valuable approach to solve these problems: so far recombinant laccases have been produced in *Saccharomyces cerevisiae* (5, 19, 21, 22), *Pichia pastoris* (9, 18, 26, 30, 37), *Yarrowia lipolytica* (17), *Trichoderma reesei* (20), *Aspergillus oryzae* (3, 36), *Aspergillus sojae* (15), *Aspergillus niger* (25, 34), and *Aspergillus nidulans* (25).

Filamentous fungi are, at least so far, the best producers of recombinant laccases, with protein yields from 70mg/l (34) to 230mg/l (20). However, considering the manipulations required to produce laccases with desirable physicochemical characters (higher redox potential, more neutral optimal pH, and better thermostability), filamentous fungi are probably not the organisms of choice for expression. To this aim, the easily manipulable yeasts are more cost-effective hosts, as demonstrated by the expression of *Myceliophthora thermophila* laccase and its directed evolution in *S. cerevisiae*, recently reported by Bulter *et al*. (4).

Kluyveromyces lactis, a promising yeast specie, has been shown to secrete a number of eucaryotic proteins far more efficiently than *S. cerevisiae* (16) with reported yields ranging from 20 mg l^{-1} to a few grams per liter (2). *K. lactis* has additional features that make it an attractive alternative to *S. cerevisiae* for foreign gene expression and secretion, including a faster growth rate, a wider substrate range, a lack of hyperglycosylation, and a reduced catabolite repression (16).

In this paper we describe for the first time the expression of recombinant *P. ostreatus* laccases POXC and POXA1b in the yeasts *K. lactis* and *S. cerevisiae* in order to compare their ability in laccase production. The *K. lactis* recombinant laccases POXC and POXA1b were purified and their properties correlated with those of the native enzymes.

MATERIALS AND METHODS

Strains, media and plasmid

The *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 manipulations.

The *Kluyveromyces lactis* strain used for heterologous expression was CMK5 (**a** *thr lys pgi1 adh3 adh1::URA3 adh2::URA3*) (35).

The plasmid pYG132 (35) was engineered from pKD1, a natural plasmid originally isolated from *Kluyveromyces drosophilarum* (8) that can stably replicate in *K. lactis*. Expression of inserts is controlled by the ethanol-inducible KlADH4 promoter (28) and the *S. cerevisiae* phosphoglycerate kinase (PGK) terminator.

The *Saccharomyces cerevisiae* strain used for heterologous expression was W303-1A (MAT ade2-1, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, can1-100).

The plasmids used for *S. cerevisiae* expression were pAC1 (*ADH1* constitutive promoter), pSAL4 (copper-inducible *CUP1* promoter) and YEp352 (*PGK1* constitutive promoter). Both strain and plasmids were kindly gifted by Dr. T. Tron (LBS CNRS UMR 6517 Faculté des Sciences de St Jérôme, Marseille, France).

E. coli was grown in LB medium (10 g l^{-1} bacto tryptone, 10 g l^{-1} NaCl, 5 g l^{-1} yeast extract), supplemented, when required, with 100 μg ml^{-1} of ampicillin.

K. lactis was grown in different media, supplemented, when required, with 100 μg ml^{-1} of geneticin G418: YPPD (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); YPD (10 g l^{-1} yeast extract, 20 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); YPPG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} galactose); YPPDG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose, 20 g l^{-1} galactose).

S. cerevisiae was grown in selective medium (6.7 g l^{-1} yeast nitrogen base w/o amino acids and ammonium sulfate; 5 g l^{-1} casaminoacids; 30 mg l^{-1} adenine; 40 mg l^{-1} tryptophane; 50 mM succinate buffer pH 5.3) containing glucose or galactose: SD (selective medium containing 20 g l^{-1} glucose); Sd (selective medium containing 10 g l^{-1} glucose); SG (selective medium containing 20 g l^{-1} galactose); Sg (selective medium containing 10 g l^{-1} galactose).

Vectors construction

The GeneBank accession numbers of the sequences of the *Pleurotus ostreatus* laccase cDNAs *poxc* (11) and *poxa1b* (12) reported in this paper are Z34848 and AJ005018 respectively.

The expression vectors were constructed using a PCR cloning approach using *Pfu* polymerase (Stratagene), and the cloned PCR products were checked by sequencing. Table 1 shows the primers used in the PCR experiments and the corresponding annealing temperatures.

The 5' regions of both cDNAs were amplified with Fwpoxc/Revpoxc and Fwpoxa1b/Revpoxa1b primers, using pUC13 containing POXC cDNA (Cpox13) and pUC18 containing POXA1b cDNA (A1bpox18) respectively as templates in order to insert a Hind III site (Fig. 1A and Fig. 2A, step 1). The Hind III DNA fragments containing the laccase encoding sequences, including their signal peptides, were ligated into pYG132 (Fig. 1A and Fig. 2A, step 2), giving pYCps and pYA1bps vectors. With the aim to insert the laccase cDNAs in the expression vectors pAC1 and pSAL4, the 3' regions of both cDNAs were amplified with FwEcoRV/Crev and FwBglII/A1brev primers that allowed to insert an EcoRI sequence (Fig. 1A and Fig. 2A, step 3). The EcoRI DNA fragments containing the laccase encoding sequences, including their signal peptides, were ligated into the expression vectors (Fig. 1A and Fig. 2A, step 4), giving C-pAC1, C-pSAL4, B-pAC1 and B-pSAL4 vectors.

Vectors where the endogenous signal peptide coding sequences were replaced with the *K. lactis* killer toxin (α-subunit) (38) or the *S. cerevisiae* invertase (*SUC2*) leader sequences were constructed (Fig. 1B, C and Fig. 2B). A synthetic DNA fragment corresponding to the pre-sequence (16 aa) of the *K. lactis* killer toxin (α-subunit) was opportunely ligated to the DNA fragment

carrying the *poxc* mature cDNA (Fig.1B). A HindIII fragment containing the *K. lactis* killer toxin sequence in frame with the *poxc* cDNA was subcloned into pYG132, giving pYCtk vector.
The 5' regions of both mature cDNAs were amplified with Cfw/Revpoxc and A1bfw/Revpoxa1b primers, using Cpox13 and A1bpox18 respectively as templates (Fig. 1C and Fig. 2B). The NsiI/HindIII fragments containing the invertase leader sequence in frame with the cDNAs were subcloned into YEp 352, giving C-YEp and B-YEp vectors.
All the described above vectors as well as the control vectors were used to transform the yeasts.

Yeast transformation, cultivation and laccase production

K. lactis transformation was performed by electroporation with a Bio-Rad Micro-Pulser apparatus, as specified by the manufacturer. The cells were spread on YPPD medium containing 100 μg ml^{-1} of geneticin G418, after an over night incubation at 28°C in YPPD. Agar plate assays on YPPD supplemented with 100 μg ml^{-1} of geneticin G418, 2% ethanol, 0.1 mM $CuSO_4$ and 0.2 mM ABTS were used for transformant selection. Plates were incubated inverted for 4 days at 28°C and checked for the development of green colour. 100 μl of ethanol was added each day to the lid of the plate to compensate its evaporation. 30 transformed clones for each expression vector were screened for laccase production by growing them in 10 ml selective medium (YPPD containing geneticin) supplemented with 0.1 mM $CuSO_4$ and 2% ethanol, at 28°C on a rotary shaker (150 r.p.m.). 2% ethanol was daily added and samples were taken at intervals for optical density and laccase activity determination. The best producing clones were chosen to study laccase production. Inocula grown on selective YPPD medium were used to seed flasks (250 ml) containing 50 ml of selective medium, starting from 0.06 OD_{600}. Cultures were grown at 28°C on a rotary shaker for 4 days. Preparative culture was performed in 1l flasks containing 200 ml of medium.
S. cerevisiae transformation was performed by using the lithium acetate procedure (13). The cells were spread on selective medium and plates were incubated inverted for 4 days at 28°C. Production of laccase by transformants was first assayed on SD plates supplemented with 0.6 mM $CuSO_4$ and 0.2 mM ABTS. Inocula grown on selective SD medium were used to seed flasks (250 ml) containing 50 ml of selective medium, starting from 0.5 OD_{600}. Cultures were grown at 28°C on a rotary shaker at 150 r. p. m. Preparative culture was performed in 1l flasks containing 200 ml of medium.

Assay of laccase activity

Culture aliquots (1 ml) were daily collected and cells were removed by centrifugation (12000 g for 2 min 4°C). Laccase activity in the culture supernatant was assayed at 25°C, monitoring the oxidation of ABTS at 420 nm (ε_{420}= 3.6 x 10^4 M^{-1} cm^{-1}): the assay mixture contained 2 mM ABTS, 0.1 M Na-citrate buffer, pH 3.0.
Laccase activity towards DMP was assayed in a mixture containing 1 mM DMP and the McIlvaine's citrate-phosphate buffer adjusted to pH 5.3. Oxidation of DMP was followed by an absorbance increase at 477 nm (ε_{477}= 1.48 x 10^4 M^{-1} cm^{-1}).

Electrophoresis and isoelectric focusing (IEF)

Polyacrylamide (9%) gel slab electrophoresis in 0.1% SDS was carried out as described by Laemmli (24). Cell lysates were obtained resuspending cellular pellets in SDS loading buffer and incubating them for fifteen minutes at 90°C, vortexing each five minutes to help cell disruption.
Analytical IEF in the pH range 3.5-9.5 was performed on 4.6% acrylamide gel slab with a Multiphor electrophoresis system (Amersham Biosciences), according to the manufacturer's instructions.
Native PAGE was performed at alkaline pH under non-denaturing conditions. The separating and the stacking gels contained 9% and 4% acrylamide, respectively. Buffer solution used for the separating gel contained 50 mM Tris-HCl (pH 9.5), and 18 mM Tris-HCl (pH 7.5) for the stacking gel. The electrode reservoir solution was 25 mM Tris-HCl and 190 mM glycine (pH 8.4). Gels were stained to visualize laccase activity by using ABTS as the substrate.

Western blotting

Proteins were separated on SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (Immobilion P, Millipore). Electroblotting was performed in 10 mM CAPS (3-(cyclohexylamino)-1-propanesulfonic acid), pH 11.0, 10% (v/v) methanol at 50 V for 180 min at room temperature. Washing solution was 5% (w/v) dried milk, in phosphate-buffered saline solution supplemented with 0.2% (v/v) Triton X100 (washing buffer). The membrane was washed and incubated with rabbit anti-POXC antibodies diluted 1:5000 or rabbit anti-POXA1b antibodies diluted 1:2000 in washing buffer at room temperature for 1 h, under continuous shaking. Subsequently, the membrane was washed and incubated as above described with anti-rabbit IgG, peroxidase conjugate (Sigma), diluted 1:2000 in washing buffer. Blots were visualised with 100 mM Tris-HCl, pH 7.5, 0.5 mg ml^{-1} 3,3'-diaminobenzidine, 0.03% (w/v) $NiCl_2$, 0.006% (v/v) H_2O_2.

Laccase purification

K. lactis culture media were harvested on the optimal laccase production day, cells were sedimented by centrifugation at 12000 g at 4°C for 15 min. 1 mM PMSF was added to culture supernatant, and secreted proteins were precipitated by the addition of $(NH_4)_2SO_4$ up to 80% saturation at 4°C and centrifuged at 10000 g for 40 min. After this step, different purification strategies were exploited for rPOXA1b and rPOXC.

The rPOXA1b ammonium sulphate precipitate was resuspended in 50 mM Na-phosphate pH 6.0, and, after extensive dialysis against the same buffer, loaded onto a SP Sepharose Fast Flow (Amersham Biosciences) column equilibrated with the phosphate buffer. After washing, the enzyme was eluted at a flow rate of 1 ml min^{-1} with a linear gradient from 0 to 0.4 M NaCl. The active fractions were pooled, concentrated and desalted on an Amicon PM-30 membrane.

The rPOXC ammonium sulphate precipitate was resuspended in 50 mM Na-phosphate pH 7.0 containing 1 M $(NH_4)_2SO_4$, and loaded onto a Phenyl Sepharose High Performance 35/100 column (Amersham Biosciences) equilibrated with the same buffer. After an isocratic step at 1 M $(NH_4)_2SO_4$, a linear gradient from 1 to 0 M $(NH_4)_2SO_4$ was applied. The active fractions were pooled, concentrated and desalted on an Amicon PM-30 membrane. Then, the sample was loaded onto a Q Sepharose Fast Flow (Amersham Biosciences) column equilibrated with the phosphate buffer and eluted from ionic exchange chromatography at a flow rate of 1 ml min^{-1} with a linear gradient from 0 to 0.4 M NaCl. The active fractions were pooled, concentrated and desalted as above.

Protein determination

Protein concentration was determined using the BioRad Protein Assay (BioRad), with BSA as standard.

Reduction and alkylation of cysteine residues

Mass spectrometry analyses were performed on the Comassie blue-stained proteins (0.4 nmol) excised from a preparative SDS electrophoresis on a 9% polyacrylamide gel. Excised bands were washed with acetonitrile and then with 0.1 M ammonium bicarbonate. Protein samples were reduced, by incubation in 10 mM dithiothreitol for 45 min at 56°C, and carboxamidomethylated by using 55 mM iodoacetamide in 0.1 M NH_4HCO_3 for 30 min, in the dark, under nitrogen atmosphere at room temperature.

Mass spectrometry analyses were also performed on the soluble recombinant proteins (1 nmol) reduced in Tris 300 mM pH 8.0, EDTA 20 mM (containing guanidinium chloride 6 M) by incubation with a 10:1 molar excess of dithiothreitol over the protein SH groups at 37°C for 2h. The free cysteine residues were alkylated by using a 5:1 excess of iodoacetamide over the total SH groups at room temperature for 1h in the dark under a nitrogen atmosphere. Protein samples were freed from salt and reagent excess by passing the reaction mixture through a PD10 pre-packed column (Pharmacia Biotech), equilibrated and eluted in 0.4% ammonium bicarbonate, pH 8.5.

Enzymatic hydrolysis

The gel particles were washed with ammonium bicarbonate and acetonitrile. Enzymatic digestions were carried out with trypsin (10 μg ml^{-1}) in 50 mM ammonium bicarbonate, pH 8.5 at 4°C for 2h.

The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 18h at 37°C. Minimum reaction volume, sufficient for complete rehydratation of the gel, was used. Peptides were then extracted, washing the gel particles with 20 mM ammonium bicarbonate and 0.1% trifluoroacetic acid in 50% acetonitrile at room temperature and then lyophilised. Aliquots of the digests were directly analysed by MALDI-MS (Matrix Assisted Laser Desorption Ionisations Mass Spectrometry). The tryptic peptide mixture was deglycosylated with peptide N-glycosidase F (PNGaseF) by incubation overnight in 0.4% ammonium bicarbonate, pH 8.5 at 37°C. Aliquots of the digest were analysed directly by MALDI MS. V8, trypsin and AspN digestions in solution were performed on carboxamidomethylated laccase samples in 0.4% ammonium bicarbonate, pH 8.5 at 37°C for 18h using an enzyme/substrate ratio of 1:50.

Mass spectrometry analysis

MALDI mass spectra were recorded using a Voyager DE an Voyager DE Pro MALDI-TOF mass spectrometer (Applied Biosystems); a mixture of analyte solution, α-cyano-4-hydroxy-cinnamic acid or di-hydroxy-benzoic as matrices, bovine insuline and horse heart myoglobin as standards were applied to the sample plate and air-dried. Mass calibration was obtained using the quasi-molecular ions (MH^+) from horse myoglobin (16,952.50 *m/z*), bovine insuline (5734.59 *m/z*), and α-cyano-4-hydroxy-cinnamic acid (379.06 *m/z*) as internal standards. Raw data were analysed by using computer software provided by the manufacturer and are reported as average or monoisotopic masses.

RESULTS

Two laccase isoenzymes secreted by *P. ostreatus*, POXC (31) and POXA1b (12), have been heterologously expressed in *K. lactis* and *S. cerevisiae.* The sequence of the amplified *poxc* cDNA cloned into the expression vectors exactly matched the deposited sequence, whilst DNA sequencing of the amplified *poxa1b* cDNA, as well as of three other clones tested, revealed one nucleotide deviating from the deposited sequence in position 687. This mutation generated one amino acid substitution, involving residue 201 of the mature protein (codon GAC instead of AAC, coding D instead of N).

K. lactis transformants.

The production of recombinant laccases was first assayed on plate using ABTS as substrate. Several transformants (85%) carrying pYA1bps vector gave colour change after 3 days, whilst no colour change was observed either for transformants carrying the pYCps or the pYCtk vectors, both containing the *poxc* cDNA.

30 different recombinant clones from each transformation were grown in liquid cultures and their laccase production was tested. The amount of extracellular laccase activity produced after 3 days by the pYA1bps transformants ranged from 0 to 9 x 10^{-2} U ml^{-1}, whilst activities ranging from 0 to 6 x 10^{-4} U ml^{-1} were measured for pYCps transformants. On the other hand, no laccase activity and no signal on western blot analyses of cellular extracts and culture supernatants was detected, either in pYCtk transformants or in control cultures. The best producing clones (HpYC for r-POXC and HpYA1b for r-POXA1b) were selected to study the time course of laccase production and to characterise the recombinant enzymes. Western blot analyses of cellular extracts and culture supernatants showed that the majority of the produced recombinant proteins were secreted (Fig. 3A-1 and 3B-1). Moreover, the protein recognised by the anti-POXA1b antibodies exhibited a slightly higher apparent molecular mass (Mr) than that of the native protein, whilst the protein recognised by the anti-POXC antibodies had a Mr very close to that of the native laccase.

Cultures of HpYC and HpYA1b clones were carried out to improve laccase production varying the growth conditions (inducer concentration, medium composition, copper concentration). The selected transformants were first grown in YPPD geneticine medium in the presence of 0.1 mM $CuSO_4$ and variable ethanol (inducer) concentration. Both HpYC and HpYA1b produced the highest laccase activity (0.007 U ml^{-1} and 2.03 U ml^{-1} respectively) in the presence of 0.5% ethanol. In Fig. 4A results are reported normalised respect to culture growth (mU/OD_{600}). Indeed, the presence of 2% ethanol reduced cell growth of 50% after 4 days.

Influence of the media composition on laccase production was then evaluated in the presence of 0.5% ethanol and 0.1 mM $CuSO_4$. HpYC and HpYA1b were cultivated in different rich media containing glucose, galactose or glucose and galactose. The highest secreted laccase activities (0.067 U ml^{-1} and 3.9 U ml^{-1} respectively) were obtained in YPPG medium (Fig. 4B).

Copper concentration was then varied in the YPPG geneticine 0.5% ethanol medium. rPOXC activity was detected in the medium even in absence of copper, but the laccase activity increased as the copper concentration increased. A concentration of at least 0.5 mM $CuSO_4$ was required to produce the highest rPOXC activity (0.1 U ml^{-1}). On the other hand, rPOXA1b activity appeared not to be strictly dependent on copper concentration, as there was not any noticeable increase in the secreted activity (4.2 U ml^{-1}) with the increase in copper concentration (Fig. 4C). Cell growth was not affected by copper, in fact biomass yield was almost identical for both transformants at all copper concentrations. No variation of the amount of produced enzymes was observed by Western blot analysis changing copper concentration (data not shown).

S. cerevisiae transformants.

Two *S. cerevisiae* clones (C-YEp and C-pSAL4) out of the three transformed with vectors containing the *poxc* cDNA, and all *poxa1b* transformants were found positive to plate assay. Judging by both the intensity of the colour, and the diameter of the halo, there was a marked difference in the expression of laccases either among the different constructs used or between the two laccases heterologously expressed. The yeasts transformed with plasmids derived from pSAL4

were the best laccase producers for both *P. ostreatus* laccases, and all the *poxa1b* transformants gave better results than *poxc* transformants. Similar results were obtained in liquid cultures, and therefore further investigations were only performed on *S. cerevisiae* transformed with plasmids derived from pSAL4. The amount of rPOXA1b (0.2 U ml^{-1}) secreted activity was significantly higher than that of rPOXC (5.4 x 10^{-4} U ml^{-1}).

Western blot analyses conducted on cellular extracts and culture supernatants of two rPOXA1b producing clones (C-pSAL4 and B-pSAL4) showed that most of the produced recombinant protein was localised inside the cell (Fig. 3A-2 and 3B-2). Moreover, the secreted rPOXA1b exhibited a higher apparent Mr than that of the native protein (likely hyper-glycosylated), whilst the protein recognised by the anti-POXC antibodies in both compartments had a Mr very close to that of the native laccase.

Cultures were carried out to improve laccase production varying growth conditions, with a particular attention to the effect of inducer and nutrients. The promoter used is inducible by copper, which is also the laccase cofactor. The selected transformants were first grown in SD medium containing $CuSO_4$ at different concentration. B-pSAL4 produced the highest laccase activity (0.2 U ml^{-1}) in the presence of 0.6 mM $CuSO_4$ (Fig. 5A). On the other hand, very low laccase activity was detected in the case of C-pSAL4, and no reliable values for production could be determined. As earlier observed for *K. lactis*, copper did not affect *S. cerevisiae* cell growth.

Due to the low amount of recombinant protein produced, influence of media composition on laccase production was evaluated only for yeast cells transformed with B-pSAL4, being rPOXC secreted activity too low. B-pSAL4 was cultivated in different selective media containing glucose or galactose in the presence of 0.6 mM $CuSO_4$. The highest activity of secreted recombinant laccases (0.2 U ml^{-1}) was obtained in medium containing glucose (Fig.5B).

rPOXA1b and rPOXC purification and characterisation

Purification and characterization of both recombinant enzymes was carried out from *K. lactis* cultures, as the production of secreted laccase activity in *S. cerevisiae* was too low.

HpYA1b transformant (the best rPOXA1b producing clone) was grown in 1l of selective YPPG supplemented with 0.5% ethanol and 0.5 mM $CuSO_4$ for 3 days. The enzyme was purified from the culture broth by ammonium sulphate precipitation (80%) and elution at 0.28 M NaCl from a cation exchange chromatography (Tab. 2A). The sample was found to be homogeneous on SDS-PAGE on which the recombinant protein behaved as a 71kDa protein whereas native POXA1b migrated as a 62kDa protein (12) (Fig. 6A). When analysed by IEF, the recombinant protein showed two equally represented bands at pH 7.2 and 7.3, whilst the native POXA1b showed several bands in the pH range of 6.5 ÷ 7.6 (data not shown).

The kinetic constants determined for rPOXA1b are reported in Table 3A. No marked differences between the native and the recombinant POXA1b were observed when DMP was used as substrate. On the other hand, it is worth noting that K_M towards ABTS of the recombinant protein was about one order of magnitude lower than that of the native protein, whereas k_{cat} were similar.

Mass spectrometry analyses of rPOXA1b allowed to validate 83% of the total amino acid sequence. The signal peptide was correctly cleaved, whilst the C-terminus was mapped as peptide 497-511 (1536.90 m/z), indicating that two amino acids from the C-terminus were cleaved. rPOXA1b amino acid sequence contained five potential N-glycosylation sites (N-X-S/T): three of them (N294, N434 and N470) were found glycosylated in *K. lactis*. The oligosaccharide chains linked to the pentasaccharide core ranged from five to eight mannose residues for N294, from one to ten mannose residues for N434, and from zero to four mannose residues for N470. No information was found regarding the residue N342, whilst N490 was found not modified. Native protein shows N294, N470 and N490 with up to five mannose residues linked to the pentasaccharide core, N434 not modified, whilst no information was available regarding residue N342 (12).

rPOXC was purified from the culture broth of HpYC transformant grown in the same conditions described for HpYA1b (Tab. 2B). Ammonium sulphate precipitation, hydrophobic interaction chromatography, and anionic exchange chromatography were sequentially used. The active fraction

eluted at 0.28 M NaCl from Q-Sepharose chromatography appeared to be homogeneous on SDS-PAGE and the recombinant protein migrated with an apparent Mr similar to that of the native POXC (Fig. 6B). Recombinant and native proteins displayed the same mobility when analysed by native PAGE and IEF (data not shown).

The kinetic constants determined for rPOXC are reported in Table 3B. As earlier observed for rPOXA1b, rPOXC K_M towards ABTS was about one order of magnitude lower than that of the native protein, whilst K_M towards DMP was similar. On the other hand, k_{cat} was significantly lower than that of the native enzyme, when both substrates were considered. No variation of specific activity was observed after incubation of rPOXC at room temperature with copper present as Cu(II) (5 mM $CuSO_4$) or Cu(I) (5mM $CuSO_4$ in the presence of 0.1, 0.5, 1 mM sodium ascorbate) (25).

Mass spectrometry analyses of rPOXC allowed the validation of 70% of the amino acidic sequence. rPOXC signal peptide was correctly cleaved, but no information regarding the C-terminal sequence could be obtained. POXC amino acidic sequence contains two potential N-glycosylation sites, one of them (N444) found glycosylated in *K. lactis*. The oligosaccharide chains linked to the pentasaccharide core ranged from three to seven mannose residues. In the native protein the same site was found glycosylated with up to six mannose residues linked to the pentasaccharide core (31).

DISCUSSION

The first heterologous expression of a functional laccase was described at the beginning of the nineties (22). From then, although many laccase genes have been cloned and sequenced from several fungi, only few laccases have been successfully expressed in heterologous hosts. In this paper we report, for the first time, the heterologous expression of *P. ostreatus* laccases POXC and POXA1b in the budding yeast *K. lactis*, comparing its performances with those of *S. cerevisiae* expressing the same laccases.

Laccase expression in yeasts has been performed using both native laccase or yeast signal peptides (*S. cerevisiae* α-factor, invertase) and different results were reported for various expressed enzymes. In *P. pastoris* better results were obtained with the laccase leader peptide as compared to a *S. cerevisiae* α–factor fusion (18, 26, 37), whereas other authors reported comparable extracellular activities via the two signal peptides (30). In *S. cerevisiae*, a higher secreted activity was found using the yeast α-factor leader peptide (19) or the yeast invertase leader peptide (21). Therefore, no univocal results were obtained and the best performing leader peptide to drive the secretion of recombinant laccases in yeasts is not predictable.

For the recombinant expression of POXC in *K. lactis* both the laccase signal peptide and the killer toxin leader sequence were tested and intra and extra-cellular productions of laccase were analysed. No production of the recombinant enzyme in both compartments was detected by immunoblot analysis when the killer toxin leader sequence was used. On the other hand, laccase signal peptide allowed the production and the secretion of active rPOXC in the medium. These data indicates that the leader peptide not only affects the secretion, but also the production of the recombinant enzyme.

For the heterologous expression in *S. cerevisiae* three expression vectors were constructed for each protein, differing for replication origins, promoter regions and leader sequences. The plasmid with 2μ as replication origin, copper inducible *CUP1* promoter region, and homologous laccase signal peptides gave the best results for both laccases. Since not all the possible combinations were exploited, no general conclusion can be inferred, even if the native signal peptides seem to be more efficient in producing extracellular *P. ostreatus* laccases, either in *K. lactis* or *S. cerevisiae*.

One nucleotide in *poxa1b* cDNA used for the expression deviates from the known genomic sequence: this substitution, found in all the clones sequenced, exchanged an N for D in position 201, and removed one of the six potential N-glycosylation sites. The amplified cDNA may be a natural allelic variant, since the *P. ostreatus* strain used was a dikaryon.

Levels of laccase secreted activity were markedly different: rPOXA1b transformants always gave higher activity than rPOXC transformants, and production of both laccases in *S. cerevisae* was significantly lower than that in *K. lactis*. The same results were obtained for all the *S. cerevisiae* vectors in all growth conditions tested (i. e. inducer concentration, medium composition, copper concentration). Furthermore, the apparent molecular mass of the recombinant proteins expressed in *K. lactis* were similar to those of the native ones, whereas *S. cerevisiae* recombinant proteins showed higher molecular masses. These data confirm the low tendency of *K. lactis* respect to *S. cerevisiae* to hyperglycosylate recombinant proteins.

K. lactis recombinant laccases were purified to homogeneity, characterized, and their properties compared to those of the corresponding native proteins. Amounts of the purified rPOXA1b and rPOXC were similar (1.1 and 1.4 mg l^{-1}, respectively, taking into account the purification yield), but rPOXC specific activity is about two orders of magnitude lower than that of the native one, whereas no significant difference was observed between rPOXA1b and native POXA1b specific activities. When the effect of copper concentration on *K. lactis* laccase production was evaluated, rPOXC showed a positive correlation between copper availability and laccase activity up to 0.5 mM $CuSO_4$, higher $CuSO_4$ concentration having no further effect. Moreover, no activity increase was observed when purified rPOXC was incubated with either Cu(II) or Cu(I) (25). These results led to hypothesize that rPOXC undergoes an incomplete or incorrect folding not strictly dependent on copper availability.

K. lactis recombinant enzymes showed slightly higher molecular masses than the native ones, with a more marked difference in the case of POXA1b (11.3% by SDS-PAGE) than POXC (1.6% by SDS-PAGE). Mass spectrometry analyses allowed to validate the primary structures of *K. lactis* rPOXA1b and rPOXC, and to determine their glycosydic moiety structures. POXC contains two potential N-glycosylation consensus sites in its sequence, one of them being modified both in the recombinant and in the native protein. On the other hand, POXA1b contains more potential N-glycosylation consensus sites in its sequence respect to POXC, and three of them were found modified in the recombinant protein as well as in the native one. Consequently, glycosylation extent per site, rather than number of N-glycosylated sites, should affect molecular mass of the heterologously expressed enzymes.

It is worth to note that rPOXA1b undergoes a C-terminal processing, as well as the native protein (12). Indeed, rPOXA1b was found depleted of the last two amino acids, whilst a C-terminus heterogeneity was observed for the native POXA1b. C-terminal processing at a conserved processing site has been reported for ascomycete laccases, and a role of the C-terminus in modulating enzymatic activity has been hypothesised (14, 19). Further investigations are needed to evaluate the functional role of POXA1b C-terminal processing.

Data reported confirmed *K. lactis* as an efficient recombinant protein producer, and proved it as a suitable system for fungal laccase production. Availability of such a recombinant expression system could allow us a deeper understanding of the laccase function-structure relationships, as well as to develop new oxidative catalysts using molecular evolution techniques.

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FIGURE LEGENDS

Fig. 1 Construction of *poxc* expression vectors. (A) pYCps: pYG132 vector containing *poxc* cDNA with its leader sequence; C-pAC1: pAC1 vector containing *poxc* cDNA with its leader sequence; C-pSAL4: pSAL4 vector containing *poxc* cDNA with its leader sequence. (B) pYCtk: pYG132 vector containing *K. lactis* killer toxin leader sequence. (C) C-YEp: YEp352 vector containing *S. cerevisiae* invertase leader sequence.
Selection markers, promoter regions and replication origins are indicated. The hatched region encodes the native signal peptide, the grey region the killer toxin leader peptide or invertase leader peptide. Fwpoxc/Revpoxc, FwEcoRV/Crev, Cfw/Revpoxc are the primers used to amplify *poxc* cDNA. *H*, HindIII; *E*, EcoRI; *K*, KpnI; *X*, XbaI; *EV*, EcoRV; *N*, NsiI.

Fig. 2 Construction of *poxa1b* expression vectors. (A) pYA1bps: pYG132 vector containing *poxa1b* cDNA with its leader sequence; B-pAC1: pAC1 vector containing *poxa1b* cDNA with its leader sequence; B-pSAL4: pSAL4 vector containing *poxa1b* cDNA with its leader sequence. (B) B-YEp: YEp352 vector containing *S. cerevisiae* invertase leader sequence.
Selection markers, promoter regions and replication origins are indicated. The hatched region encodes the native signal peptide, the grey region the invertase leader peptide. Fwpoxa1b/Revpoxa1b, FwBglII/A1brev, A1bfw/Revpoxa1b are the primers used to amplify *poxa1b* cDNA. *H,* HindIII; *E*, EcoRI; *B*, BglII; *Bh*, BamHI, *N*, NsiI.

Fig. 3 Western Blot analyses of cellular extracts and culture supernatants. (A) rPOXC expressing clones: 1, *K. lactis* clone HpYC; 2, *S. cerevisiae* clone C-pSAL4. (B) rPOXA1b expressing clones: 1, *K. lactis* clone HpYA1b; 2, *S. cerevisiae* clone B-pSAL4.

Fig. 4 Effect of growth conditions on secreted laccase activities from *K. lactis* normalised respect to culture growth. (A) Inducer (ethanol) effect. Cultures are grown in YPPD selective medium containing 0.1 mM $CuSO_4$ and different ethanol concentration. □, 0% EtOH; ■, 0.5%EtOH; ■, 1%EtOH; ■, 2%EtOH. (B) Nutrient effect. Cultures are grown in selective medium supplemented with 0.5% ethanol and 0.1mM $CuSO_4$. □, YPPD (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); ■, YPD (10 g l^{-1} yeast extract, 20 g l^{-1} bacto tryptone, 20 g l^{-1} glucose); ■, YPPG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} galactose); ■, YPPDG (10 g l^{-1} yeast extract, 40 g l^{-1} bacto tryptone, 20 g l^{-1} glucose, 20 g l^{-1} galactose). (C) Copper effect. Cultures are grown in YPPG medium supplemented with 0.5% ethanol and different copper concentration. □, 0mM $CuSO_4$; ■, 0.1mM $CuSO_4$; ■, 0.3mM $CuSO_4$; ■, 0.5mM $CuSO_4$; , 1mM $CuSO_4$.

Fig. 5 Effect of growth conditions on secreted laccase activities from *S. cerevisiae* normalised respect to culture growth. (A) Inducer (copper) effect. Cultures are grown in SD medium containing different copper concentration. □, 0.3 mM $CuSO_4$; ■, 0.6 mM $CuSO_4$; ■, 1.2 mM $CuSO_4$; ■, 1.8 mM $CuSO_4$. (B) Nutrient effect. Cultures are grown in selective medium supplemented with 0.6 mM $CuSO_4$. □, SD (selective medium containing 20 g l^{-1} glucose); ■, Sd (selective medium containing 10 g l^{-1} glucose); ■, SG (selective medium containing 20 g l^{-1} galactose); ■, Sg (selective medium containing 10 g l^{-1} galactose).

Fig. 6 SDS-PAGE analyses of rPOXA1b (A) and rPOXC (B). Lane 1 recombinant protein; lane 2 native protein; lane 3 molecular mass standards.

Fig. 7 Amino acid sequence of POXA1b and POXC. The underlined stretches have been verified by MALDI MS for the recombinant proteins. Putative N-glycosylation sites are in bold; sites found glycosylated are indicated by an asterisk.

TABLE 1. List of the primers used in the amplification experiments. Nucleotides in bold are complementary to the laccase cDNA sequences. Nucleotides in italics are recognized by restriction enzymes.

Primer	Nucleotide sequences	ann. T
Fwpoxc	TTT*GAATTCAAGCTT***ATGTTTCCAGGCGCACGG**	60°C
Revpoxc	**GGCCACGATAT*GGTACC*AATCC**	60°C
Fwpoxa1b	ATAA*GAATTCAAGCTT***ATGGCGGTTGCATTCG**	54°C
Revpoxa1b	**GTGTGCCCGACA*AGATCT*GCAACAAC**	54°C
A1bfw	AAAA*GAATTCATGCAT*CT**AGCATTGGGCCCCGCGG**	54°C
A1brev	TAA*GGATCCGAATTC***TTATAATCATGCTTTCAATGGC**	54°C
FwBglII	**TGTTGC*AGATCT*TGTCGG**	54°C
Cfw	AAAA*GAATTCATGCAT*CT**GCCATTGGGCCCGCTGGC**	60°C
FwEcorV	**AGCC*GATATC*AACATCAATCTCG**	60°C
Crev	AAAA*TCTAGAGAATTC***TTAAGAAGTAGGAATAAGAAG**	60°C

TABLE 2. (A) rPOXA1b and (B) rPOXC purification from *Kluyveromyces lactis* cultures.

A

Purification Step	**Total activity**	**Total protein**	**Specific activity**	**Recovery**	**Purification Fold**
	U	**mg**	**U/mg**	**%**	
Broth	1392	94.7	14.7	100	1.0
$(NH_4)_2SO_4$ precipitate	910	32.0	28.4	65.4	1.9
S Sepharose	745	0.6	1307.0	53.5	88.9

B

Purification Step	**Total activity**	**Total protein**	**Specific activity**	**Recovery**	**Purification Fold**
	U	**mg**	**U/mg**	**%**	
Broth	54.6	106.0	0.51	---	1.0
$(NH_4)_2SO_4$ precipitate	73.2	100.8	0.73	100	1.4
Phenyl Sepharose	59.3	3.8	15.6	81.1	30.6
Q Sepharose	59.1	1.1	51.8	80.7	101.6

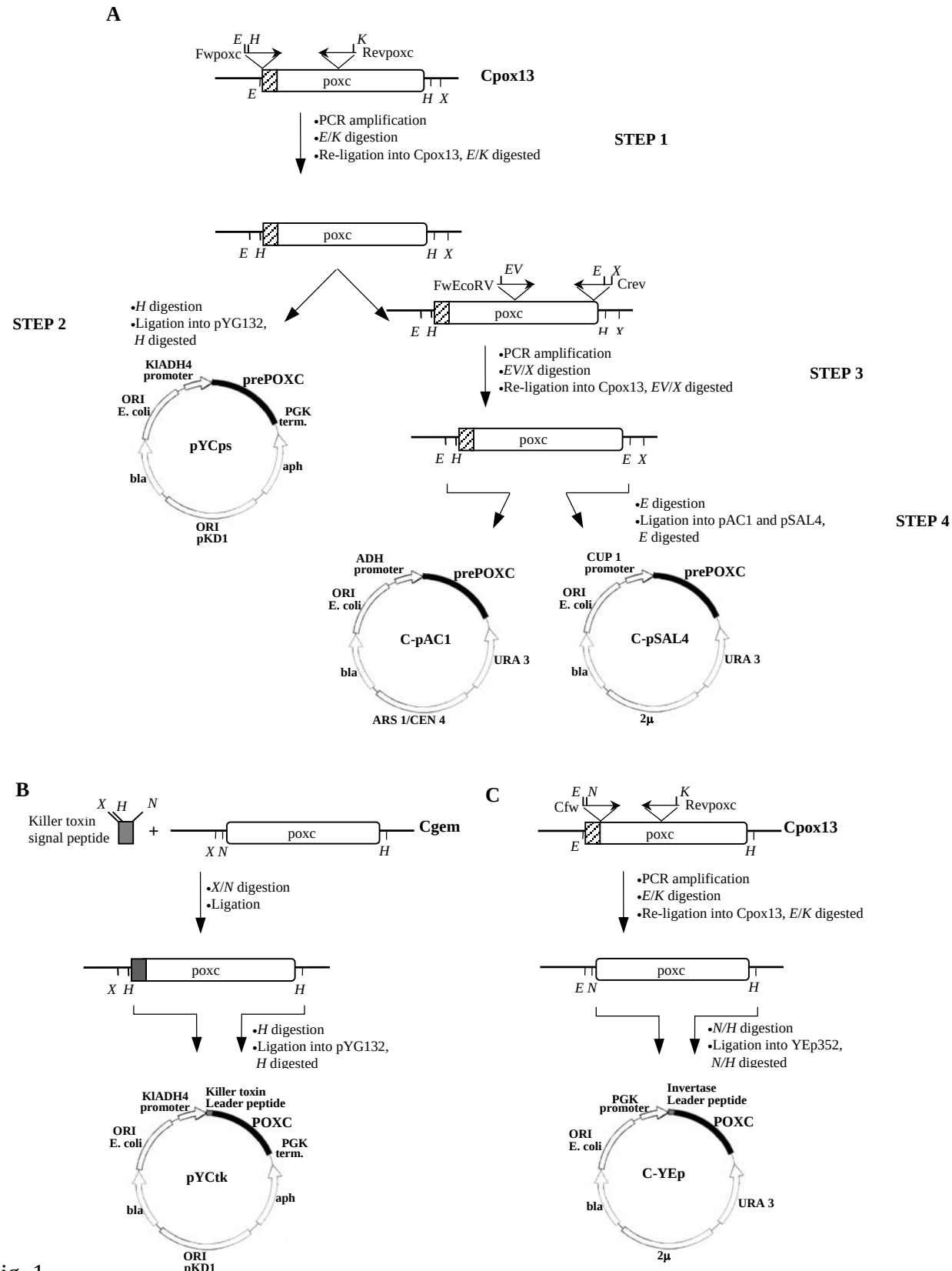
TABLE 3. (A) rPOXA1b and (B) rPOXC kinetic constants compared to those of the native proteins.

A

Substrate	**K_M (mM)**		**k_{cat} (min^{-1})**	
	POXA1b	**rPOXA1b**	**POXA1b**	**rPOXA1b**
ABTS	0.47±0.06	0.04±0.01	$(9.0\pm1.7)\times10^4$	$(9.0\pm1.2)\times10^4$
DMP	0.26±0.09	0.29±0.04	$(1.5\pm0.1)\times10^4$	$(5.5\pm0.7)\times10^4$

B

Substrate	**K_M (mM)**		**k_{cat} (min^{-1})**	
	POXC	**rPOXC**	**POXC**	**rPOXC**
ABTS	0.28±0.04	0.03±0.01	$(1.4\pm0.2)\times10^5$	$(3.2\pm0.4)\times10^3$
DMP	0.07±0.01	0.13±0.02	$(4.8\pm0.2)\times10^5$	$(1.0\pm0.1)\times10^3$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E H
Fwpoxc
K
Revpoxc
poxc
Cpox13
E
H X
•PCR amplification
•E/K digestion
•Re-ligation into Cpox13, E/K digested
STEP 1
poxc
E H
H X
STEP 2
•H digestion
•Ligation into pYG132,
H digested
EV
E X
FwEcoRV
Crev
poxc
E H
H X
•PCR amplification
•EV/X digestion
•Re-ligation into Cpox13, EV/X digested
STEP 3
KlADH4 promoter
prePOXC
ORI E. coli
PGK term.
pYCps
aph
bla
ORI pKD1
poxc
E H
E X
•E digestion
•Ligation into pAC1 and pSAL4,
E digested
STEP 4
ADH promoter
prePOXC
ORI E. coli
C-pAC1
URA 3
bla
ARS 1/CEN 4
CUP 1 promoter
prePOXC
ORI E. coli
C-pSAL4
URA 3
bla
2µ
B
X H N
Killer toxin signal peptide
+
poxc
Cgem
X N
H
•X/N digestion
•Ligation
poxc
X H
H
•H digestion
•Ligation into pYG132,
H digested
KlADH4 promoter
Killer toxin Leader peptide
POXC
ORI E. coli
PGK term.
pYCtk
aph
bla
ORI pKD1
C
E N
Cfw
K
Revpoxc
poxc
Cpox13
E
H
•PCR amplification
•E/K digestion
•Re-ligation into Cpox13, E/K digested
poxc
E N
H
•N/H digestion
•Ligation into YEp352,
N/H digested
PGK promoter
Invertase Leader peptide
POXC
ORI E. coli
C-YEp
URA 3
bla
2µ

Fig. 1

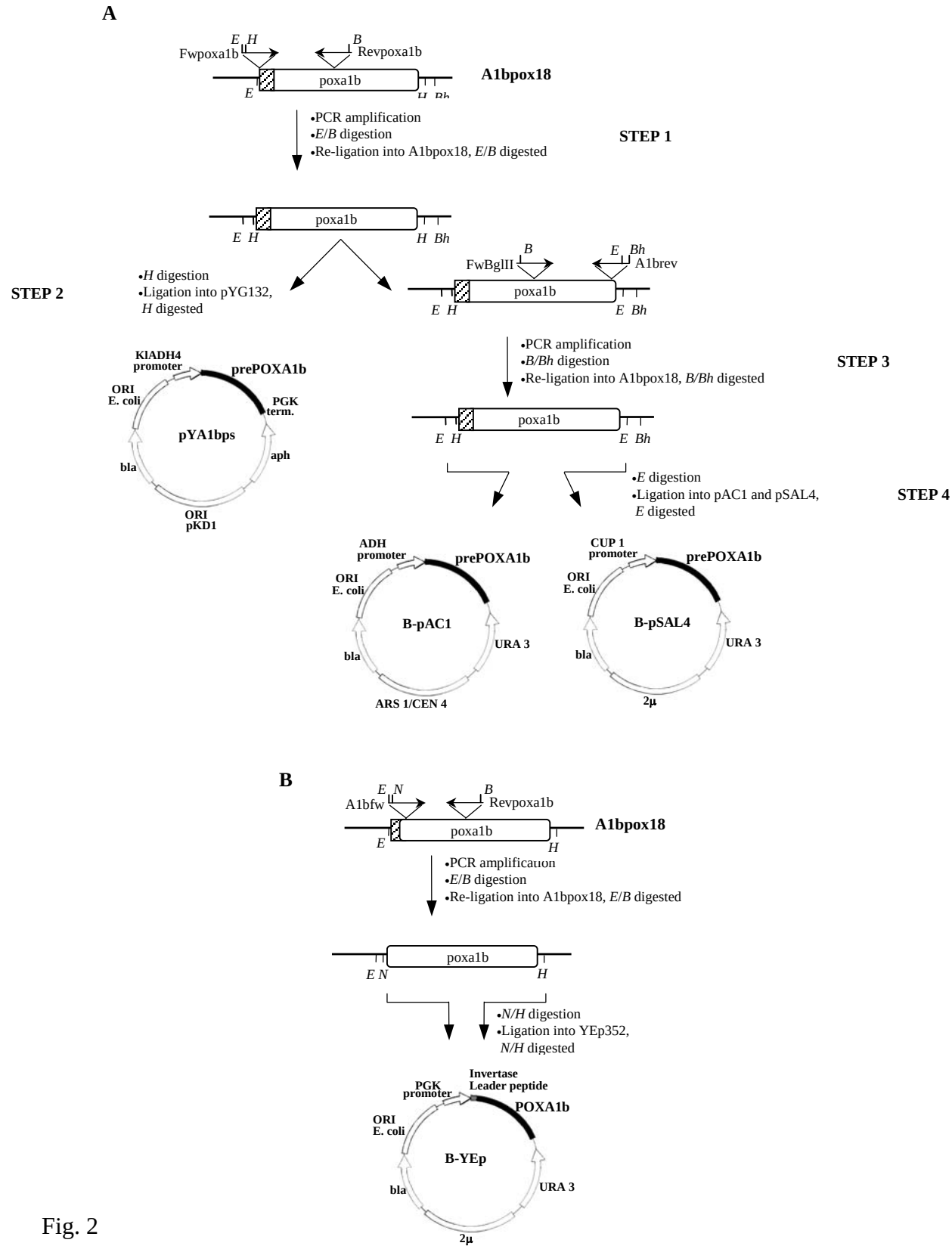
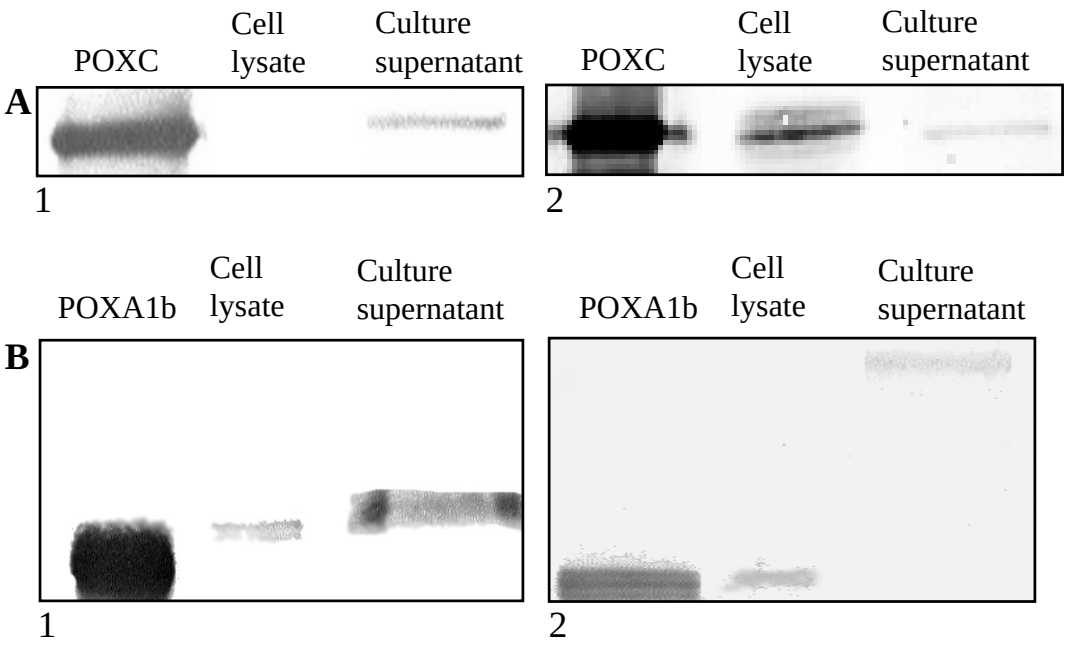

A
E H
Fwpoxa1b
B
Revpoxa1b
poxa1b
E
H Bh
A1bpox18
•PCR amplification
•E/B digestion
•Re-ligation into A1bpox18, E/B digested
STEP 1
poxa1b
E H
H Bh
STEP 2
•H digestion
•Ligation into pYG132,
H digested
B
FwBglII
E Bh
A1brev
poxa1b
E H
E Bh
•PCR amplification
•B/Bh digestion
•Re-ligation into A1bpox18, B/Bh digested
STEP 3
poxa1b
E H
E Bh
•E digestion
•Ligation into pAC1 and pSAL4,
E digested
STEP 4
KlADH4 promoter
prePOXA1b
ORI E. coli
PGK term.
pYA1bps
aph
bla
ORI pKD1
ADH promoter
prePOXA1b
ORI E. coli
B-pAC1
URA 3
bla
ARS 1/CEN 4
CUP 1 promoter
prePOXA1b
ORI E. coli
B-pSAL4
URA 3
bla
2µ
B
E N
A1bfw
B
Revpoxa1b
poxa1b
E
H
A1bpox18
•PCR amplification
•E/B digestion
•Re-ligation into A1bpox18, E/B digested
poxa1b
E N
H
•N/H digestion
•Ligation into YEp352,
N/H digested
Invertase Leader peptide
PGK promoter
POXA1b
ORI E. coli
B-YEp
bla
URA 3
2µ

Fig. 2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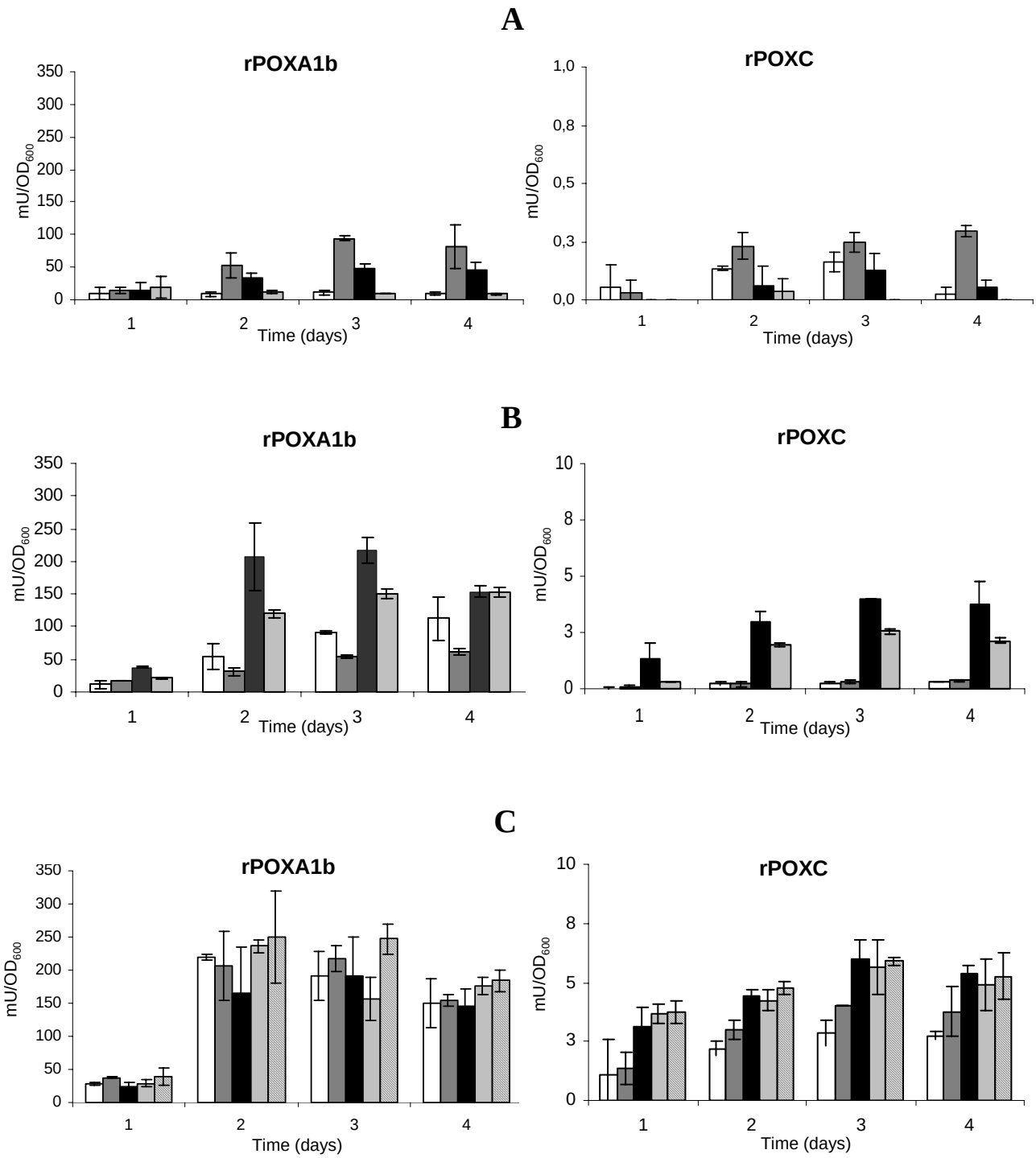
POXC
Cell lysate
Culture supernatant
1
POXC
Cell lysate
Culture supernatant
2
B
POXA1b
Cell lysate
Culture supernatant
1
POXA1b
Cell lysate
Culture supernatant
2

Fig. 3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rPOXA1b
mU/OD600
350
300
250
200
150
100
50
0
1
2
3
4
Time (days)
rPOXC
1,0
0,8
0,5
0,3
0,0
B
10
8
5
3
C

Fig. 4

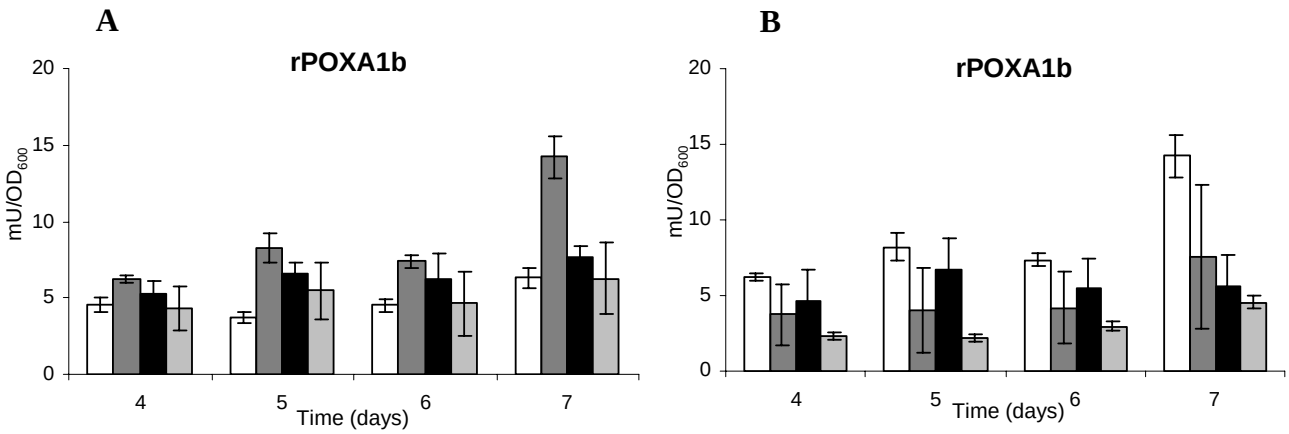
A
rPOXA1b
mU/OD600
20
15
10
5
0
4
5
6
7
Time (days)
B
rPOXA1b
mU/OD600
20
15
10
5
0
4
5
6
7
Time (days)

Fig.5

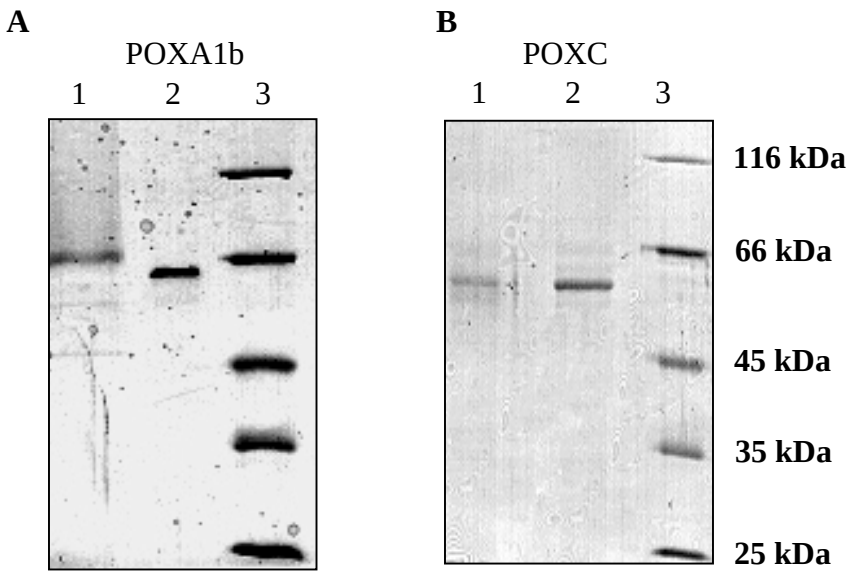

Fig. 6

P0XA1b

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RGPLVVYSKN DPHKRLYDVD DESTVLTVGD WYHAPSLSLT GVPHPDSTLF NGLGRSLNGP 180
ASPLYVMNVV KGKRYRIRLI DTSCDSNYQF SIDGHTFTVI EADGENTQPL QVDQVQIFAG 240
                                                        *
QRYSLVLNAN QAVGNYWIRA NPNSGDPGFE NQMNSAILRY KGARSIDPTT PEQNATNPLH 300
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RDAHDLAPAG SIYDIKLGDV VEITMPALVF AGPHPIHLHG HTFAVVRSAG SSTYNYENPV 420
          *                                             *
RRDVVSIGDD PTDNVTIRFV ADNAGPWFLH CHIDWHLDLG FAVVFAEGVN QTAAANPVPE 480
AWNNLCPIYN SSNPSKLLMG TNAIGRLPAP LKA                            513
```

P0XC

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ITVDSIQIFA GQRYSFVLTA NQAVDNYWIR ANPNLGSTGF VGGINSAILR YAGATEDDPT 300
TTSSTSTPLL ETNLVPLENP GAPGPPVPGG ADININLAMA FDFTTFELTI NGVPFLPPTA 360
PVLLQILSGA STAASLLPSG SIYELEANKV VEISMPALAV GGPHPFHLHG HTFDVIRSAG 420
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SISAPPAAWD DLCPIYNALS DNDKGGIVPS                                510
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Fig.7