Engineering of small laccase (SLAC) from *Streptomyces coelicolor* for application in biocatalysis and surface functionalisation

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DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the award of the Doctor of Philosophy in Biotechnology, to the Durban University of Technology, Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.

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DEDICATION

Dedicated to Almighty God and My Family

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Research outputs:

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Conferences:

SASM (The South African Society of Microbiology), 2018: Secretory expression of SLAC from the *Streptomyces coelicolor* in *Pichia pastoris*, its characterisation and application in dye decolorisation. (Oral presentation). https://www.sasm2018.co.za/

ICCBN (Third **International Conference on Composites, Biocomposites and Nanocomposites), 2018:** Modified magnetic nanoparticles as a novel carrier for the immobilisation of small laccase: characterisation and application in the removal of phenol and its derivatives (Oral presentation). https://www.dut.ac.za/iccbnm/

Abstract

The small laccase (SLAC) from *Streptomyces coelicolor* A3(2) is a versatile and industrially relevant biocatalyst mainly because of desirable characteristics such as activity at high temperatures and alkaline pH and relative stability against common laccase inhibitors. However, low yield from natural hosts, low catalytic efficiency and lack of reusability are some of the technological barriers that tend to limit the industrial application of the SLAC. In the present study, strategies have been developed to engineer SLAC for the improvement of catalytic properties and expression of SLAC for potential application in biocatalysis and surface functionalisation.

Site directed mutagenesis was used to enhance properties of the enzyme. Four mutant sites (Y229A, Y230A, V290N and M198G) were selected on the basis of some positive results from literature and from the four sites, ten composite mutants including double, triple and quadruple mutants were generated. The produced variant SLAC enzymes were purified to homogeneity by Ni^{2+} affinity chromatography in a single step. A double mutant (Y230A/V290N) showed a low K_m value which was 75% lower than wild type small laccase (WT-SLAC). Double mutations (Y230A/V290N and Y229A/M198G) enhanced the ability of the enzyme to decolourise common industrial dyes. For example, the double mutant Y229A/M198G exhibited a 1.2-fold higher catalytic efficiency (9.12 $min^{-1}mM^{-1}$) for the oxidation of ABTS than the WT-SLAC (7.46 $min^{-1}mM^{-1}$) and was able to decolourise 50 mg/L of Methyl Red (MR) completely, whilst only 28% decolourisation was observed with the WT-SLAC.

To enhance enzyme production, the SLAC (from *S. coelicolor* A3(2)) was expressed in the methylotrophic yeast *Pichia pastoris*. The SLAC gene was cloned under the control of methanol inducible alcohol oxidase 1 (AOX1) promoter. The recombinant *P. pastoris* yielded

high titres of extracellular laccase (500 ± 10 U/L) upon induction with methanol. The extracellular SLAC (~38 kDa) was purified to homogeneity with a specific activity of 8916.66 U/mg. The purified SLAC had an optimum activity at 80 C, but optimum pH varied with substrate used (pH 4 for ABTS and pH 8 for syringaldazine (SGZ) and 2,6-dimethoxy-phenol (2,6-DMP). K_m values for ABTS, SGZ and 2,6-DMP were 142.85 μM, 10 μM and 54.55 μM and the corresponding k_{cat} values were 60.6 s^{-1}, 25.36 s^{-1} and 27.84 s^{-1}, respectively. The $t_{1/2}$ values of the recombinant SLAC (rSLAC) at 60 ºC, 70 ºC, 80 ºC were found to be 60 h, 32 h and 10 h, respectively. The enzyme deactivation energy (E_d) was 117.275 kJ/mol while ΔG, ΔH and ΔS for thermal inactivation of the rSLAC were all positive.

To further enhance enzyme production, a dual promoter system was investigated using a combination of AOX (inducible promoter) and GAP (constitutive promoter). To this end, a recombinant *P. pastoris* strain harbouring rSLAC under control of both AOX and GAP promoters, was generated. A production level of 1800 U/L was recorded for rSLAC-GAP-AOX which was about 3.6-fold higher than the single promoter system (rSLAC-AOX). Mixed feed strategy (9:1 ratio of methanol/glycerol) led to a positive influence in biomass accumulation and 1.5-fold increase in rSLAC expression, compared with induction of the double promoter system with methanol alone.

To facilitate reusability of SLAC, the enzyme was immobilised onto surface of silanized magnetic nanoparticles (Si-MNP's). Briefly, a silane layer was coated using 3-(aminopropyl) triethoxysilane (APTES) over the surface of MNPs, which was followed by immobilisation of rSLAC via covalent attachment. Field emission-scanning electron microscopy (FE-SEM) images of MNPs revealed that the diameter of the MNPs was in the range of 50-200 nm. Fourier transform-infrared spectroscopy (FT-IR) spectra of MNP, MNP/APTES and

MNP/APTES/rSLAC, confirmed the presence of characteristic peaks at 589 cm^{-1}, 2360 cm^{-1} and 1648 cm^{-1}, respectively. MNP-rSLAC showed remarkable storage stability (retained ≥95% of initial activity after storage in Tris-HCl buffer 20 mM, pH 8 at 4 °C over a period of 30 days), temperature stability, and tolerance towards organic solvents and heavy metals. Repeated usage of MNP-rSLAC showed >73% of its initial activity after 10 catalytic cycles and the enzyme was easily recovered from the reaction mixture by the application of a magnetic field.

The potential application of rSLAC was investigated in the degradation of several pollutants and in surface functionalisation. rSLAC efficiently decolourised two synthetic dyes tested belonging to triphenylmethane and azo group of dyes. More than 90% decolourisation was achieved for Brilliant Blue G and Trypan Blue in 6 hours without the assistance of any mediator. Several phenolic pollutants such as phenol, 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP), categorised as "priority pollutants", were completely degraded within 2 hours using only 2 U of rSLAC. Growth inhibition studies using *Escherichia coli* showed that rSLAC-mediated treatment of phenolic compounds reduced the toxicity of phenol, 4-CP and 4-FP by 90, 60 and 55%, respectively. In addition, ciprofloxacin and tetracycline, two of the most persistent classes of drugs were also degraded by rSLAC in combination with acetosyringone (AS) as mediator. rSLAC-AS degraded 95% of 5 mg/L ciprofloxacin (CIP) and 100 % of 150 mg/L of tetracycline (TC) within 6 hours. The removal of TC resulted in complete elimination of antibacterial activity while up to 48% reduction in antibacterial activity was observed when CIP was removed.

The rSLAC also catalysed the functionalisation of two biological materials (chitosan and coconut fibres) to improve antimicrobial properties. The rSLAC oxidised functional molecules

to corresponding radicals which reacted with the lignin moieties and amino groups of the coconut fibres and chitosan, respectively. The appearance of broad absorption band around 380 and 450 nm of the UV/Vis spectra of grafted chitosan films, indicated a reaction between o-quinone and amino groups of chitosan. FT-IR spectrum of grafted biomaterials showed new aromatic skeletal vibrations as well as phenolic absorption bands indicating conjugation of allelochemicals onto chitosan and coconut fibres. Antimicrobial activities of grafted biomaterials were found to be up to 60% higher than that of their ungrafted counterparts.

In conclusion, the SLAC from *S. coelicolor* A3(2) was engineered to enhance its catalytic properties and expressed extracellularly in *P. pastoris*. With combined use of constitutive and inducible promoters, production level reached up to 1800 U/L, which was among the highest yields attained for recombinant bacterial laccases expressed in *P. pastoris*. This study has shown that a combination of site-directed mutagenesis, secretory expression and immobilisation could lead to the production of a viable rSLAC for application in bioremediation and surface functionalisation.

PREFACE

This thesis is structured into nine chapters. The first chapter gives the general introduction to the thesis. The second chapter is a review of literature relevant to the study. Chapter three presents the work done on engineering of the small laccase (SLAC) from *Streptomyces coelicolor* by site-directed mutagenesis. Chapter four presents the secretory expression of SLAC in *Pichia pastoris.* Chapter five explores the use of dual a promoter system in *Pichia pastoris* for the enhancement of the production levels of the recombinant small laccase (rSLAC). Chapter six presents the immobilisation of rSLAC on magnetic nanoparticles (MNPs). Chapter seven reports the application of rSLAC in the surface modification of coconut fibres and chitosan with the allelochemicals. Chapter eight is a general discussion of the findings of the present study. Chapter nine concludes the thesis and suggests recommendations for further studies.

CHAPTER 1

General Introduction

The interest in enzymes as alternatives to chemical catalysts has been increasing in recent years. One enzyme that has gained considerable attention is laccase (EC 1.10.3.2). This is mainly due to its broad substrate specificity (Wang and Zhao, 2017), an aspect which has been exploited in various biotechnological applications such as decolourisation of effluents, polyphenol removal in beverages, and bioremediation (Mollania *et al.*, 2011). Laccases isolated from mesophilic fungi (Sherif *et al.*, 2013) have been extensively exploited industrially (Prins *et al.*, 2015) due to their high redox potential. However their use is restricted towards mesophilic temperature ranges and acidic pH conditions (Gunne and Urlacher, 2012). In addition, post-translational challenges (such as intron processing and glycosylation) complicate the heterologous expression of fungal laccases (Prins *et al.*, 2015). Typical industrial process conditions can also affect the large scale application of laccases (Sherif *et al.*, 2013). Enzymatic properties such as catalytic efficiency, redox potential, enzyme production yield, thermostability, tolerance towards a wide pH range and organic solvents, need to be enhanced to facilitate the application of laccases at industrial level. However, the presence of all the favourable characteristics in a single laccase is highly unlikely (Gupta *et al.*, 2019). Therefore, researchers are now also considering bacterial laccases as possible alternatives to fungal laccases.

Interest towards bacterial laccases is growing owing to their atypical characteristics (Prins *et al.*, 2015). Laccases from bacteria offer several potential benefits such as high stability at a wider pH range, thermostability, as well as tolerance towards common inhibitors. In addition, prokaryotic systems can be used for enzyme production, and genetic manipulation is relatively

easy (Gunne and Urlacher, 2012). One such laccase is the small laccase (SLAC) from *Streptomyces coelicolor* A3(2), which is a two-domain multi-copper oxidase that lacks the second domain typically found in fungal laccases (Dubé *et al.*, 2008). However, low redox potential and low expression yield are notable drawbacks associated with bacterial laccases (Dubé *et al.*, 2008), which tend to limit their industrial application. Availability of whole genome sequences has opened up avenues to engineer the SLAC (Gupta *et al.*, 2019). Site directed mutagenesis (SDM) is one of the important tools in protein engineering which can help construct novel proteins with the aid of available SLAC gene sequence and structure. The putative key residues could be modified to generate a modified enzyme suiting the industrial needs. The variant obtained could potentially outperform the unmutated protein in terms of stability, specificity, activity, solubility, and expression levels. In addition, high-performing variants could be combined to further improve catalytic properties (Madhavan *et al.*, 2017). Furthermore, other strategies can be investigated to improve properties and enhance the production yield such as, heterologous protein expression, bioprocess optimisation, and enzyme immobilisation.

Low production levels by native microbial hosts can be problematic for commercial application (Yadav *et al.*, 2018). Heterologous production can be a feasible alternative to native hosts. Although, attempts have been made to clone the laccase-encoding gene in *Escherichia coli*, overexpression is complicated by intracellular aggregation (Yadav *et al.*, 2018) which requires downstream processing to obtain the active protein. On the contrary, extracellular expression in *Pichia pastoris* can simplify the purification steps (Yang *et al.*, 2015), as it secretes low levels of endogenous proteins (Yang *et al.*, 2015). Ability to grow at high cell densities, generation of a stable integrant, and availability of expression vectors are other advantages associated with expression in *P. pastoris* (Yadav *et al.*, 2018). Recombinant proteins can be

expressed either inducibly and/or constitutively in *P. pastoris* (Ranjan and Satyanarayana, 2016). Protein expression levels also seems to be influenced by factors such as promoter strength, codon usage, and multiple gene copies (Nordén *et al.*, 2011; Tang *et al.*, 2014). To date, no studies have been performed to express SLAC in *P. pastoris*. Use of an expression vector which utilises methanol could be promising, as methanol is inexpensive and could be used as both inducer and a carbon source. However, slow growth in methanol and low enzyme productivity could be another roadblock for effective enzyme production in a methanol induced system. Alternatively, a constitutive promoter such as $P_{GAP,}$ not only provides constitutive transcription of gene of interest (GOI), but negates the need to use a hazardous inducer such as methanol (Parashar and Satyanarayana, 2016). Since, a promoter is an essential element of an expression vector, combining strong promoters for heterologous gene expression can be a promising approach (Öztürk *et al.*, 2017). A few studies have investigated the use of double promoter systems, leading to an increase in enzyme production (Wu *et al.*, 2003; He *et al.*, 2015; Parashar and Satyanarayana, 2016). However, it is essential to meet the requirements of both promoters in order to realise the optimum potential of dual promoters (He *et al.*, 2015). The tailored transcriptional activity via strain engineering to use a double promoter system could further enhance the heterologous expression of the recombinant SLAC.

Free enzymes are often associated with numerous limitations such as large enzyme consumption, difficult separation from the reaction mixture and low recyclability (Fernandes *et al.*, 2017; Ranjan *et al.*, 2017), low stability as well as potential contamination of the free enzyme (Korecka *et al.*, 2009). Enzyme immobilisation is frequently used to counteract these drawbacks. Immobilisation on a magnetic nanoparticle (MNP) has proved to be a promising approach as the enzyme can be retrieved from the reaction mixture by simple application of a magnetic field (Pereira *et al.*, 2017; Ranjan *et al.*, 2017). However, agglomeration due to

magnetic dipole interactions (Villa *et al.*, 2016) could be a limitation associated with MNPs. Therefore, in the current study the enzyme was immobilised on MNP functionalised with an anchoring surface with selective affinity towards bio-compounds.

The combination of SDM, secretory expression using single and double promoter systems, as well as immobilisation on MNPs could enhance the utility of the SLAC in biotechnological applications.

Aim of the study

The aim of the study was to engineer the small laccase from *S. coelicolor* for application in biocatalysis and surface functionalisation.

The specific objectives were as follows:

1. To enhance the activity of SLAC by site directed mutagenesis.
2. To clone and express the SLAC gene in *P. pastoris* for secretory expression using a single and double promoter system.
3. To immobilise the SLAC on magnetic nanoparticles
4. To apply the SLAC in bioremediation and surface functionalisation.

In Chapter 2, literature relevant to the study is reviewed, while Chapter 3 reports on investigations on the site-directed mutagenesis of the SLAC for the enhancement of catalytic properties. Chapter 4 explores the secretory expression of SLAC in *P. pastoris* using a single promoter system and is followed by Chapter 5 which explores the use of a dual promoter system to enhance the production levels of SLAC in *P. pastoris.* Chapter 6 focusses on the immobilisation of SLAC on magnetic particles for the treatment of phenolic pollutants. The last experimental chapter (Chapter 7) reports on the application of the recombinant SLAC

(rSLAC) in the surface functionalisation of coconut fibres and chitosan with allelochemicals. Chapter 8 provides a general discussion of all the results obtained in the present study. The thesis ends with concluding remarks and recommendations for further studies (Chapter 9).

CHAPTER 2

Literature Review

This chapter reviews literature which is relevant to this study. Some of the areas covered in this review include: a general overview of laccases, their sources and substrate range; application of laccases; laccases as bioremediation agents and limitations of conventional waste treatment methods; current challenges limiting laccase applications; bacterial laccases; strategies for enhancing bacterial laccase feasibility as industrial catalysts; protein engineering and protein expression in *Pichia pastoris*. The literature review concludes with a scope of this study as a way of linking the literature reviewed and the current study.

2.1 Laccases, Source and Function

Industrial enzymes are expected to be highly stable and active under unfavourable environmental conditions of temperature or pH (Mollania *et al.*, 2011). Among these enzymes, laccases referred as "green oxidases" have gained considerable attention due to its broad substrate specificity (Wang and Zhao, 2017).

2.1.1 Source and function of laccases

Laccases are enzymes which are ubiquitous in nature and are widely distributed among plants, insects, archae, bacteria and fungi. Laccase was first discovered by Yoshida in 1883 in the latex of the Japanese lacquer trees (*Rhus sp.*) (Mate and Alcalde, 2015). In plants, these enzymes are involved in a number of functions including lignification, wound healing as part of an herbivore or pathogen defence response, and in iron metabolism (Hoopes and Dean, 2004). Laccases are thought to be ubiquitous among fungi, and their presence has been documented in virtually every fungus examined so far (Alessandra *et al.*, 2010). In fungi,

laccases carry out a variety of physiological roles including morphogenesis, fungal plant-pathogen/host interaction, stress defence and lignin degradation (Alessandra *et al.*, 2010). Generally, fungal laccases are monomeric globular proteins of approximately 60–70 kDa with acidic isoelectric point (pI) around pH 4.0, although several exceptions exist. Most fungal laccases are extracellular enzymes and generally glycosylated, with the extent of glycosylation usually ranging between 10 and 25%; only in a few cases is glycosylation higher than 30% (Singh *et al.*, 2011). Multiplicity of laccase genes is a common feature in fungi and plants, and the production of several laccase isoenzymes has been observed in many species (Singh *et al.*, 2011) . Now, there is increasing evidence for the existence of proteins with typical features of the multi-copper oxidase enzyme family also in prokaryotes (Singh *et al.*, 2011). Laccase-encoding genes have been found in gram-negative and gram-positive bacteria, including species living in extreme habitats. Early reports of laccases in actinomycetes were based on rather non-specific substrate reactions, but have been verified for some bacteria of the genera *Streptomyces* (Molina-Guijarro *et al.*, 2009). Physiological roles of bacterial laccases include copper homeostasis, sporulation, or pigmentation of spores to confer resistance to stress factors such as Ultra-violet (UV) radiation or hydrogen peroxide (Martins *et al.*, 2002).

The substrate range of laccases is discussed below:

2.1.2 Substrate range

Laccases are multi-copper oxidases, which are capable of catalysing one-electron oxidation of a wide range of substrates (Mate and Alcalde, 2017) mainly diphenols, methoxy substituted phenols, phenolic amines and alkylamines. They use molecular oxygen as an electron acceptor (Viswanath *et al.*, 2008), and produce only water as a by-product, hence they are generally regarded as "green catalysts". Many applications of laccases have been proposed in several industrial sectors. As a fact, laccases can be used (1) to bleach textiles (Shelke, 2001**)**; (2) to

eliminate undesirable phenolics responsible for the browning, haze formation, and turbidity development in beverages (Minussi *et al*., 2002); (3) to bleach wood pulp (Widsten and Kandelbauer, 2008)**;** (4) to synthesize various functional organic compounds such as drugs and dyes (Nicotra *et al.*, 2004; Bruyneel *et al.*, 2008); (5) to produce various polymers (Gübitz and Paulo, 2003); (6) to detect molecules in biosensor devices (Kulys and Vidziunaite, 2002); (7) to produce power in biofuel cells (Amir *et al.*, 2009) and (8) to dye hair. As far as bioremediation is concerned, laccases may be applied to decolourise textile effluents, and degrade plastic waste containing olefin units**,** to eliminate odour emitted from places such as garbage disposal sites, livestock farms, or pulp mills**,** to remove phenolic compounds from olive oil mills and pulp mills wastewaters; and to decontaminate soils from polycyclic aromatic hydrocarbons (PAHs)(Olivieri *et al.*, 2006; Kunamneni *et al.*, 2008).

Substrate oxidation by laccases takes place at the mono-nuclear copper centre (T1); electrons are then transferred to the tri-nuclear copper centre (T2/T3), where the reduction of dioxygen takes place, yielding water as the sole by-product (Quintanar *et al.*, 2007).

Due to their broad substrate specificity (Koschorreck *et al.*, 2009), laccases have generated a lot of biotechnological interest as they have application potential in different industrial fields **(Fig. 2.1)** (Mate and Alcalde, 2017). Few of the applications are discussed below in detail:

2.1.3 Application of laccases

2.1.3.1 Fibre colouration

Fibre coloration is a complex process, consisting of fibre swelling in aqueous medium, then penetration of dyes into them and its fixation. These processes require a range of dyes, pH and elevated temperatures. Oxidative dyes are applied in hair coloration and involves two-

component system-dye precursors and an oxidant such as hydrogen peroxide (Hadzhiyska *et al.*, 2006). However, hair dyeing formulations involving hydrogen peroxides are usually mutagenic. Alternatively, laccase based hair dyes are preferable as they are less irritating (Chen *et al.*, 2013). A typical laccase reaction involves the oxidation of the substrate after the transfer of a single electron to laccase, resulting in the formation of a free cation radical (Kim *et al.*, 2011). Laccase can oxidise a range of substrates from diamines, aminophenols, aminonaphtols and phenols to aryloxy-radicals. The radicals formed can further undergo a non-enzymatic reaction resulting in the formation of dimeric, oligomeric and polymeric products (Hadzhiyska *et al.*, 2006). This makes laccase suitable to be exploited in colouration reactions and be developed as a green chemistry dyeing process (Fu *et al.*, 2012).

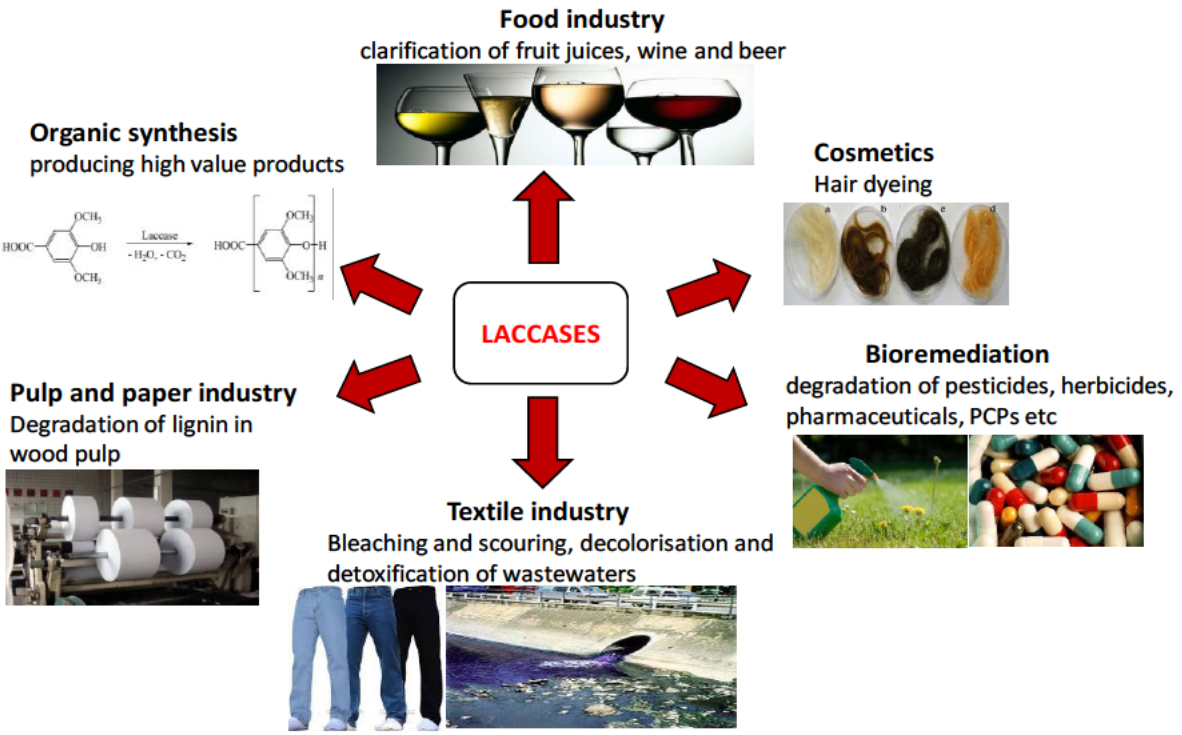


Fig. 2.1 Applications of laccases in various sectors (Chauhan *et al.*, 2017 with modifications)

2.1.3.2 Surface functionalisation

An increased consciousness towards the environment has made the development of biodegradable composites one of the hottest topics of research. Over the years there has been an increase in investigations on the applications of natural plant fibres (Wambua *et al*., 2003; Lodha and Netravali, 2005). Merits such as low cost, high availability, lightweight and high tensile strength make them a suitable candidate in natural fibre reinforced composites. However, due to their hydrophilicity and highly polar nature, they tend to show poor compatibility with hydrophobic resins (Arbelaiz *et al.*, 2006). Therefore, surface modification becomes imperative to obtain high-performance composites (Dong *et al.*, 2014). Physical (heat treatment, steam explosion, cold plasma processing and high energy ray radiation processing) and chemical (chemical initiator, high energy ray and couplers) methods have been used in various investigations to achieve surface modification (Rong *et al*., 2001; Albano *et al*., 2002; Marais *et al*., 2005; Li *et al*., 2013). However, deterioration of mechanical properties via physical methods and polymerisation of monomers via chemical methods are the shortcomings associated, which hinder the industrialization of these enzymes (Dong *et al.*, 2014). In response to the limitations associated with physicochemical methods, the use of enzymes like laccases can be the new hope for lignin valorisation (Dong *et al.*, 2014).

Lignin is a complex three-dimensional structure, mainly synthesised from three precursors (p-coumaryl alcohol, sinapyl alcohol, and coniferyl alcohol) **(Fig. 2.2)** which are linked together in an irregular fashion **(Fig. 2.3)** (Kudanga *et al.*, 2008) These monomeric units when incorporated in lignin polymer are known as p- hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Munk *et al.*, 2015; Thakur *et al.*, 2015b). The polymers are regarded as a substrate for laccase, due to the availability of numerous phenolic sites which by the action of laccase gets oxidised to phenoxy radicals, and could further couple with the oxidised phenolic molecules (Thakur *et al.*, 2015b).

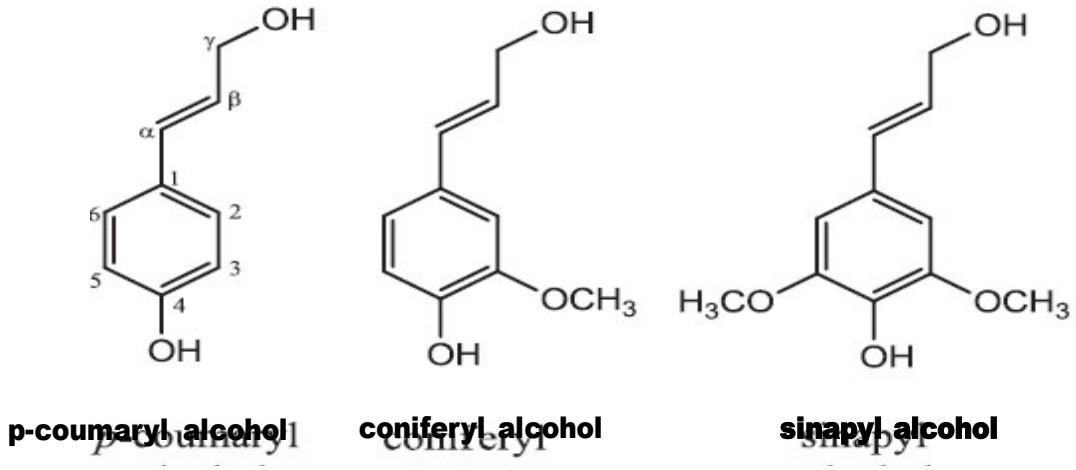


Fig. 2.2 Structure of three major lignin precursors (Munk *et al.*, 2015).

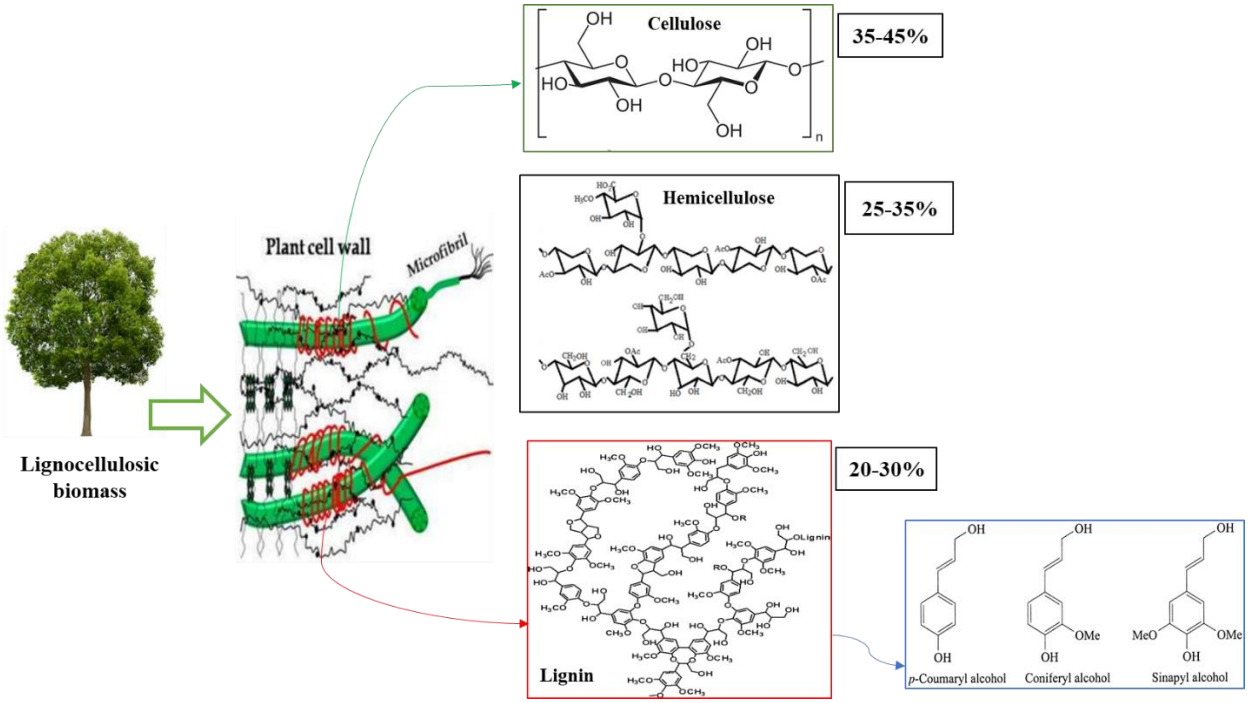


Fig. 2.3 Framework of lignocellulosic biomass with cellulose, hemicellulose, and lignin structures and percentage composition shown (Kalia *et al.*, 2014 with modifications)

Moreover, lignin can be modified by ligninolytic enzymes (such as lignin peroxidases, manganese peroxidases and laccases) (Sjöström, 1993; Boudet, 2000). With regards to hygiene standards and environment protection, enzyme-mediated grafting of antimicrobial and

hydrophobic molecules might offer a greener and valid solution in addition to improving the physio-chemical and mechanical properties of lignocellulosic materials (Thakur *et al.*, 2015a). Catalysis via laccase is in focus as it does not require the presence of hydrogen peroxide (H_2O_2), like peroxidases. The reaction mechanism of laccases uses readily available atmospheric oxygen and its simultaneous reduction to H_2O alongside the oxidation of the substrate (Munk *et al.*, 2015). Lignin oxidation via microorganisms has been extensively reported in white-rot fungi. Recently lignin oxidation by bacterial enzymes from some soil bacteria has been discovered (Bugg *et al*., 2011b; Brown and Chang, 2014) and laccase from *Streptomyces sp.* has been shown to reduce the acid precipitable lignin, and this signifies its role in lignin oxidation (Singh *et al.*, 2017). However, there remain fewer reports on the usage of bacterial laccase in lignin valorisation.

Lignocellulosics are better than traditional materials as they offer renewability, biodegradability and available at low cost (Kalia *et al.*, 2014). However, poor antimicrobial and moisture resistance are few limitations associated with lignocellulosic biomass (Elegir *et al.*, 2008; Garcia-Ubasart *et al.*, 2013). Nevertheless, the physiochemical properties of these materials can be modified by enzyme-mediated grafting (Munk *et al.*, 2015). Enzymes like laccase, peroxidase, lipases have been explored in grafting organic molecules for modifying the lignin properties (Kalia *et al.*, 2014). For example, grafting of hydroxyl groups and other polar functionalities via laccase has imparted hydrophilicity to the natural fibres (Thakur *et al.*, 2015b). The key mechanism in biografting via laccase is the "radical coupling reactions" which enables the attachment of low molecular compounds onto the lignocellulosics (Chandra *et al*., 2004; Liu *et al.*, 2009). **Fig. 2.4** illustrates different probable changes caused in lignin resulting from the action of laccase.

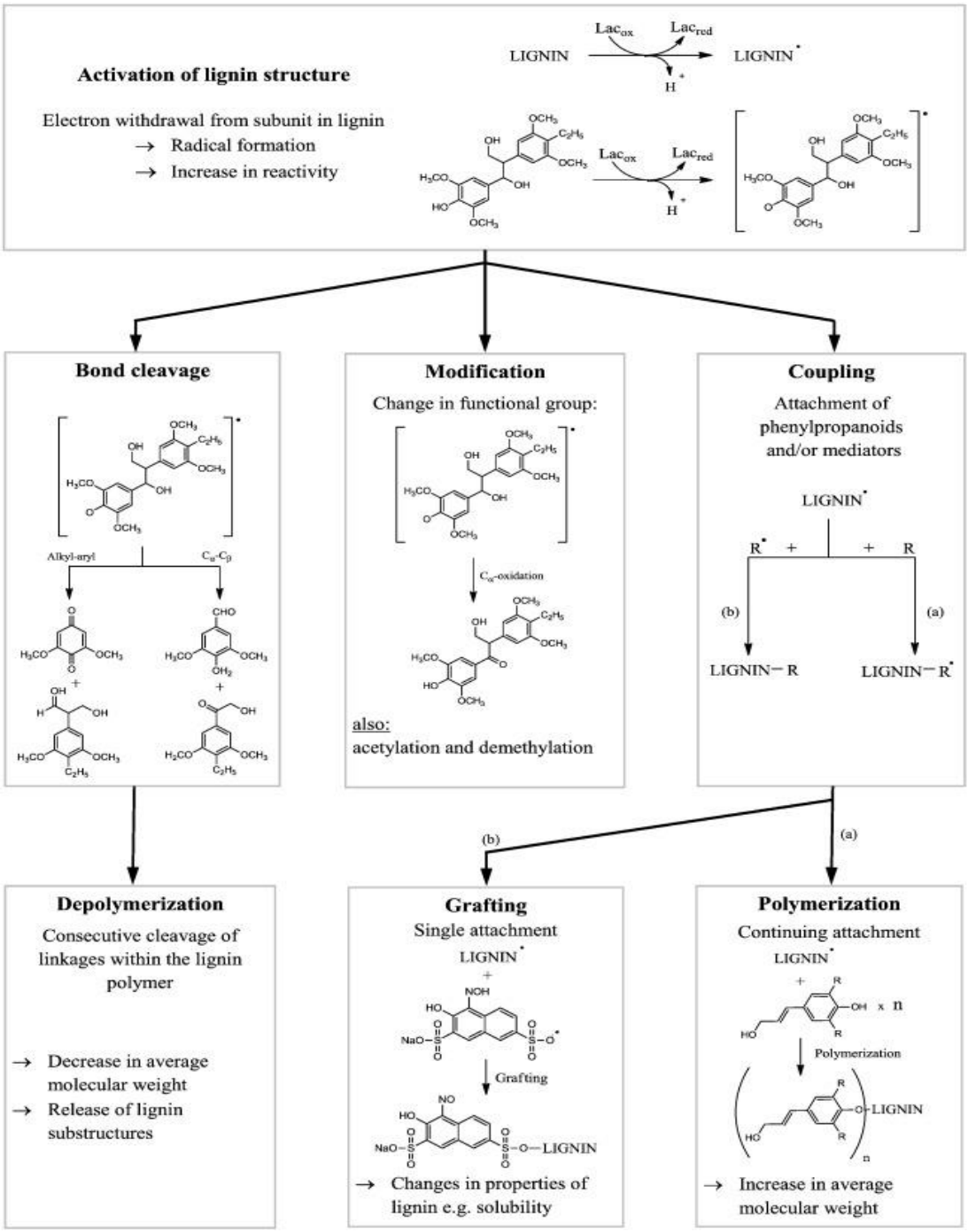


Fig. 2.4 Proposed changes due to the action of laccases on lignin. Each box demonstrates a specific kind of event (Munk *et al.*, 2015).

Different hydrophobic moieties have been introduced to reduce the hydrophilicity of the lignocellulosic materials by laccase assisted grafting **(Table 2.1)** (Kudanga, *et al.*, 2010;

Garcia-Ubasart *et al.*, 2011). Non-specific substrate requirements of laccases allow grafting of a wide range of phenolic compounds onto the fibre matrix, thereby imparting the desired properties. Numerous studies have exploited to evaluate the feasibility of this approach in functionalisation of wood materials and lignin rich fibres to impart antimicrobial and hydrophobicity properties (Aracri *et al.*, 2010). Therefore, grafting by laccases is a novel and green approach which offers innovative solutions to modify lignin rich fibres (Witayakran and Ragauskas, 2009) and to enhance the lignocellulosic performance by imparting the desired functionalities such as strength or stiffness, and resistance to microbial attack or moisture. The properties imparted can find potential applications for various fields (Kalia *et al.*, 2014). The ability of laccases to oxidise lignin has been found only in a small number of bacteria such as *Rhodococcus, Amycolatopsis, Psuedomonas* and *Streptomyces* (Granja-Travez *et al.*, 2018), which suggests their possible application in lignin valorisation (Bugg and Rahmanpour, 2015). A summary of laccase assisted grafting of functional molecules imparting various properties on lignocellulosic materials is given in **Table 2.1.**

Table 2.1 Laccase-catalysed grafting of functional molecules on different lignocellulosic materials

Lignocellulosic material	Laccase source	Functional molecule grafted	Property	References
Softwood kraft pulp	*Trametes villosa*	Amino acids	Increase in strength properties	Witayakran and Ragauskas, 2009
Cotton cellulose	*Trametes* sp.	2,5 diaminobenzenesulfonic acid and 1-hydroxyphenol (catechol)	Coloration of cotton fibres	Hadzhiyska *et al.*, 2006
dibenzodioxocin **(lignin model compound)**	*Trametes hirsuta*, *T. villosa*, *Bacillus* SF	phenolic amines, fluorophenols and selected wood preservatives	Hydrophobicity, antifungal and flame retardant properties	Kudanga *et al.*, 2010
syringylglycerol β-guaiacylether **(lignin model compound)**	*Bacillus* SF spore	Aromatic amines	leaving the $-NH_2$ group free for further functionalization	Kudanga *et al.*, 2009
guaiacylglycerol β-guaiacyl ether and syringylglycerol β-guaiacyl ether **(lignin model compounds)**	*Trametes hirsuta*	Fluorophenols	Improvement in hydrophobicity	Kudanga *et al.*, 2010
Lignin models and lignin monomers	*T. hirsuta*	long chain alkylamines	Improvement in hydrophobicity	Kudanga *et al.*, 2010
Kraft pulp	*Aspergillus oryzae*	Methyl syringate	Improvement in wet strength (wet tensile index doubled)	Liu *et al.*, 2009
Wood (spruce) chips	*T. villosa*	4-hydroxy-3-methoxybenzylamine (HMBA) or 4-hydroxy-3-methoxybenzylurea (HMBU)	Increase in internal bond	Fackler *et al.*, 2008
Kraft pulp	*T. villosa*	4-Hydroxyphenylacetic acid, 4-hydroxybenzoic acid, gallic acid, syringic acid, vanillic acid	Improving strength properties (increase in burst, tear and tensile indexes)	Chandra and Ragauskas, 2002; Chandra, 2004a, 2004b; Schroeder *et al.*, 2007
Coconut fibres	*Trametes versicolor*	Syringaldehyde	Improved mechanical and antibacterial properties	Thakur *et al.*, 2015
Coconut fibres	*T. versicolor*	*p*-coumaric acid	Improvement in hydrophobic and	Thakur *et al.*, 2015

Coconut fibres	*T. versicolor*	Ferulic acid	antibacterial properties Improvement in thermal stabilities, hydrophobic and antibacterial properties	Thakur *et al.*, 2016

Apart from lignocellulosic substrates, there has also been increasing attention of grafting phenolic acids onto chitosan to further broaden its applications (Liu *et al.*, 2017). This is due to the availability of abundant amino and hydroxyl groups found in chitosan, and hence modification with chitosan is easier than other polymers (Prabaharan, 2008).

Phenolic acids are found to enhance the bioactive properties of chitosan such as antioxidant, antimicrobial, anti-allergic, anti-diabetic, anti-inflammatory, antitumor activities (Liu *et al.*, 2017). Notably, the phenolic acid grafted chitosan can find applications as coating agent, bio-adsorbent, encapsulating agent, packing materials (Hu and Luo, 2016), food additives and dietary supplements (Hashemi Gahruie and Niakousari, 2017). **Table 2.2** shows examples of various materials grafted on chitosan. These materials are grafted using different fabrication methods such as activated ester-mediated grafting, free radical induced grafting and enzyme mediated grafting (Hu and Luo, 2016).

Table 2.2 Grafting of different functional molecules on chitosan

Molecule grafted	Grafting method	Applications	References
Gallic acid	Chemical modification by EDC/NHS; laccase-mediated method; free radical induced grafting reaction	Antioxidant additive; Anti-diabetic agent; bioactive compound delivery; food packaging; drug delivery	Yu *et al.*, 2011; Lee and Je, 2013; Liu, *et al.*, 2013; Lu, *et al.*, 2013
Caffeic acid	Chemical modification by EDC; laccase, tyrosinase-mediated method; free radical induced grafting reaction	Antioxidant additive; tissue engineering	Aytekin *et al.*, 2011; Božič *et al.*, 2012; Chiang *et al.*, 2014; Liu *et al.*, 2014
Ferulic acid	carbodiimide-mediated coupling reaction	Food additives and packaging	Woranuch and Yoksan, 2013; Woranuch *et al.*, 2015
Catechin	Redox mediated grafting	Antioxidant additive; anti-diabetic agent; iron-chelator	Brzonova *et al.*, 2011; Zhu and Zhang, 2014
Salicylic acid	Chemical modification by EDC	Antiplatelet aggregation; drug delivery; food preservative	Jiang *et al.*, 2012; Wang *et al.*, 2013
Epigallocatechin gallate (EGGE)	Free radical induced grafting reaction	Bioactive compound delivery; food additive	Lei, Liu, *et al.*, 2014; Lei, Wang, *et al.*, 2014
Eugenol	Chemical modification by CAN	Food packaging; textile material protection	Chen *et al.*, 2009; Sauperl *et al.*, 2014
phloroglucinol	Free radical induced grafting reaction	Antioxidant additive; food preservative	Woo and Je, 2013
quercetin	Chloroperoxidase-mediated method	Antimicrobial	Torres *et al.*, 2012
Tannic acid	Laccase-mediated method	Antimicrobial	Božič *et al.*, 2012

2.2 Laccases as bioremediation agents

One of the major challenges of today's civilization is environmental pollution (Rana *et al.*, 2017). Some of the pollutants such as antibiotics are a hazard to the ecosystem and pose danger to human health (Blánquez *et al.*, 2016). Wastewater generated from several industries as well as anthropogenic sources (such as hospitals) are some of the causes of global environmental problems. Some of the pollutants are discussed below:

2.2.1 Industrial pollution

2.2.1.1 Dyes

A wide range of synthetic dyes are increasingly being used by different industries and discharged, which poses a major environmental concern. Owing to complex molecular structure along with its synthetic origin (Robinson *et al.*, 2001), dyes are toxic, mutagenic, carcinogenic as well as recalcitrant molecules and are difficult to degrade biologically (Azmi *et al.*, 1998; Erkurt *et al.*, 2007). They can be classified as heterocyclic, anthraquinone, phthalocyanine, triphenylmethane, and azo, on the basis of their chemical structures (Ashrafi *et al.*, 2013). Triphenylmethane and azo dyes constitute the largest classes of commercially produced colourants. These are used in colouring paper, food, cosmetics, textiles, leather, medical treatment and analysis (Przystas *et al.*, 2012). The entrance of dyes in the aquatic system reduces light penetration, thereby reducing sunlight for photosynthetic organisms (Champagne and Ramsay, 2010) **(Fig. 2.5)**. Increasing ecological awareness and public concerns over pollution caused by dyes has led to strict legislative control in its discharge (Robinson *et al.*, 2001; Christie, 2007).

Fig. 2.5 Dye contamination caused by the release of wastewater from textile industries (Source: https://ourgoodbrands.com/guide-natural-fabric-dyes-alchemy-textile-artists/)

2.2.1.2 Phenols and their derivatives

Phenol and its derivatives can be introduced to the environment by various industries that uses them as their raw materials such as pharmaceutical, pulp, and paper, tannery, chemical, petrochemical, coal refining industries (Zhang *et al.*, 2012). Due to the toxic, carcinogenic, mutagenic and teratogenic nature of these compounds, they have been classified as priority pollutants in the US EPA list (Coniglio *et al.*, 2008). Their presence in drinking and irrigation water poses serious health and environmental hazard (Wang *et al.*, 2012).

2.2.2 Anthropogenic pollution

2.2.2.1 Pharmaceuticals

Pharmaceuticals consist of several compounds which belong to different classes such as antibiotics, analgesics, antidepressants, antiseptics, hormones, and anti-inflammatory drugs. The presence of pharmaceutical residue in the environment particularly in wastewater treatment plants (WWTPs) has already been reported by several studies (Van Doorslaer *et al.*, 2014). The degree of metabolism of the active compounds in humans could vary such that up to 90% of certain compounds could be metabolised while only 10% or less of other compounds can be metabolised. According to one report, around 70% of the total active compounds consumed in Germany remains unchanged. Most of the metabolites are more water-soluble than their parental counterpart, which leads to its excretion in urine. Furthermore, metabolism could also lead to the generation of toxic metabolites (Kümmerer, 2009). Antibiotics are widely used in human medicine and in stockbreeding operations to prevent infections and promote growth. Their ubiquitous presence is responsible for the occurrence of antibiotic resistance bacteria (ARB) in natural waters. Microbial communities acquire the antibiotic resistance against the antibiotic by one of the following ways: (1) chemical transformation by the cellular enzyme, (2) acquiring the antibiotic resistance gene from another cell via lateral gene transfer

or by the (3) activation of the efflux pumps (Vila-Costa *et al.*, 2017). Furthermore, the transfer of resistance genes in the microbiota of wastewater could lead to the emergence of deadly pathogens (Blánquez *et al.*, 2016). Antibiotic resistance has become a major health concern and has been described as one of the biggest threats (to public health) of the 21st century by the World Health Organization (WHO) (Rodriguez-Mozaz *et al.*, 2015).

All these contaminants from industries and hospitals end up in wastewater treatment plants. Consequently, the removal of these contaminants and the development of processes to remove them remains to be an important task.

2.3 Conventional wastewater treatment methods

Several physical, chemical and biological processes have been reported in the past few decades **(Fig. 2.6)** and have been accepted by textile industries **(** Saratale *et al.*, 2011**)**.

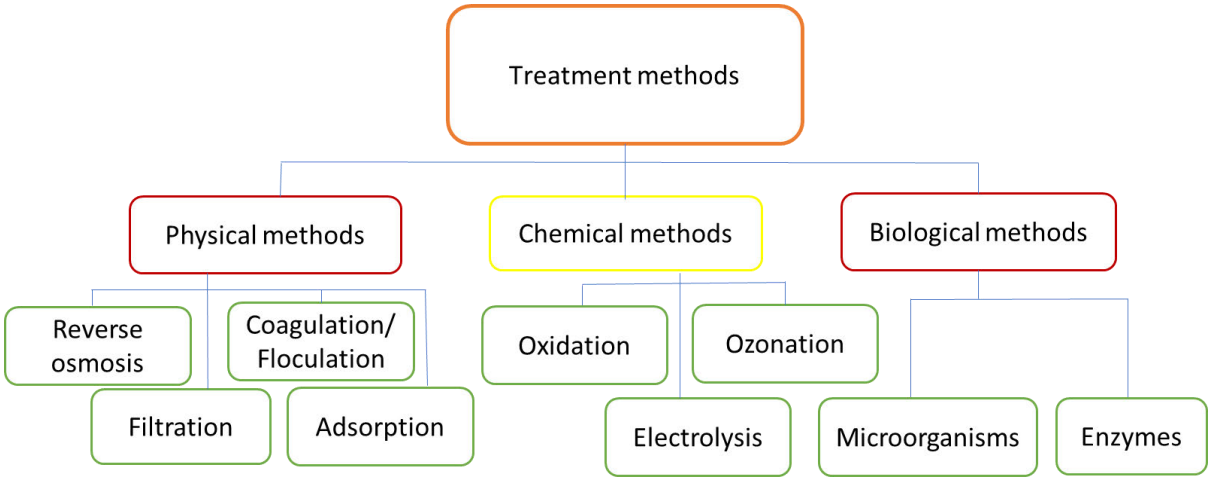


Fig. 2.6 Treatment methods for removal of dyes from wastewater effluents (Saratale *et al.*, 2011**)**

2.3.1 Physical methods

Physical methods for removal of dyes from effluents involves adsorption, reverse osmosis, coagulation or flocculation. Coagulation/Flocculation are efficient in removing sulphur and disperse dyes but inefficient in removing acid, and vat dyes. Filtration methods (such as ultrafiltration, nanofiltration and reverse osmosis), can separate hydrolysed dyestuffs which simultaneously reduces Biological oxygen demand (BOD) and chemical oxygen demand (COD) of wastewater. Depending on the wastewater chemical composition, the type and porosity of the filter is decided (Dos Santos *et al.*, 2007). Furthermore, this method clarifies, concentrates and separate dyes from effluents in a continuous fashion (Xu *et al.*, 1999). However, high investment costs, membrane fouling, secondary waste generation (which poses further disposal problems) (Robinson *et al.*, 2001; Dos Santos *et al*., 2007), membrane clogging or membrane replacement are some of the drawbacks associated with this approach. This approach can be applied to effluents consisting of low concentrations of dyes but it is inefficient in reducing the dissolved solid content, making water use difficult (Robinson *et al.*, 2001). Furthermore, removal of suspended solids (SS) at regular intervals is important to increase the lifespan of the membranes. All these problems limit the applicability of this approach in wastewater treatment (Verma, Dash and Bhunia, 2012).

Ion exchange is another physical method that is used, where wastewater is passed over the ion exchange resin until the saturation of available exchange sites and can efficiently be used for both cationic and anionic dyes. However, the use of organic solvents in regenerating the ion exchangers is cost-intensive. Also, the process is not appropriate for disperse dyes (Robinson *et al.*, 2001).

Irradiation is also another method used, but it requires sufficient quantities of dissolved oxygen to break down the organic substances. Rapid consumption of oxygen necessitates the

requirement of constant and adequate oxygen supply thereby making it an expensive approach that is not feasible in large scale operations. Electrochemical oxidation can destroy organic compounds with no release of hazardous products and is also a cost-intensive method.

Adsorption is the most common approach (Nassar and El- Geundi, 1991), this is due to its ability to efficiently remove a wide range of dyes (Saratale *et al.,* 2011). Several biological materials like low costs agricultural by-products (such as orange and cassava peel, apple pomace, wheat straw, cotton waste, rice husk, maize cob, plum kernel, etc.) have been used for dye sorption from wastewater (Sadhasivam *et al.*, 2009). In addition, bio-sorbents like seaweeds or waste generated from industries like fermentation waste activated sludge process waste has also gained considerable attention due to their high selectivity and efficiency, economical and removal performance (Aksu, 2005; Crini, 2008). Microbial biomass can prove to be an inexpensive source of sorbent that is not just capable of binding diverse species of organisms but can also be obtained as waste material from fermentation processes in industries (Kapoor and Viraraghavan, 1997; Saratale *et al.*, 2011). However, high cost, limited efficiency towards a wide range of dyes, regenerating the adsorbents or disposal problem due to the accumulation of sludge are among the problems that limit its practical application (Karcher *et al.*, 2001a; Anjaneyulu *et al*., 2005). In addition to physical methods some chemical methods are discussed below:

2.3.2 Chemical methods

Chemical methods generally involve the use of various oxidising agents such as ozone (O_3), permanganate (MnO_4) and hydrogen peroxide to alter the chemical composition of a compound. These oxidising agents require activation means such as ultraviolet light (Slokar and Majcen Le Marechal, 1998). Oxidation results in aromatic ring cleavage of the dye

molecules, thereby removing the dye from the effluents (Robinson *et al.*, 2001). Amongst all the oxidants, ozone is the strongest and has shown high reactivity and good removal efficiency with several dyes (Alaton *et al*., 2002). Ozone also doesn't increase the volume of wastewater or sludge since it is applied in gaseous form. Generally, the chromophore group in the dye is an organic compound with conjugated double bonds, which through the direct or indirect action of ozone is broken into small molecules, leading to a reduction in effluent colour (Peralta-Zamora *et al.*, 1999). However, it was inefficient with disperse and vat dyes which are non-soluble dyes and have longer reaction times (Marmagne and Coste, 1996).

In comparison to ozonation, Advanced Oxidation Process (AOP) technology (such as H_2O_2/UV process and Fenton reaction) is relatively cheaper and offers higher decolorisation efficiency as well as COD reduction. The only difference between H_2O_2/UV process and Fenton reaction is that in former case UV exposure is directed towards water containing H_2O_2 [see equation 1], whilst in later case, H_2O_2 is added in an acid solution (pH 2–3) consisting of Fe^{2+} ions [see equation 2] (Verma *et al.*, 2012).

$$H_2O_2 + UV\ (\lambda = 200\text{-}280\ nm) \rightarrow HO\cdot + HO\cdot \qquad \textbf{Equation 1}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^- \qquad \textbf{Equation 2}$$

Several drawbacks like sludge generation (resulting from flocculation of dye molecules and reagents) (Robinson *et al*., 2001), high cost and variable success (due to the dependence on the presence of various constituents in textile wastewaters) limits the application of AOP (Verma, Dash and Bhunia, 2012). A major disadvantage seems to be the short half-life (usually 20 min). The lifespan can further be affected by pH, temperature and the presence of salts. In addition

to high cost, the need for continuous ozone supply (Xu *et al.*, 1999) and constant monitoring of effluent pH (Slokar and Majcen Le Marechal, 1998) makes AOP an inefficient approach.

Electrochemical destruction is a relatively new technique that is significantly effective in dye removal as there is no chemical consumption and build-up of the sludge. Also, the metabolites generated aren't hazardous therefore the treated wastewater can be released into the natural environment. However, the high cost of electricity limits the use of this process (Saratale *et al.*, 2011).

Chlorine compounds have also been used in chemical oxidation. The chlorine cation (Cl^+) attacks the amino group of the dye molecule which initiates and accelerates the cleavage of the azo bond. The rate of decoloration increases with an increase in chlorine concentration and decreasing the pH of the medium (Anjaneyulu *et al*., 2005). However, due to the negative effects of chlorine (Slokar and Majcen Le Marechal, 1998) and carcinogenic and toxic nature of aromatic amines; and the fact that this approach is not suitable for disperse dyes, its use is less frequent (Robinson *et al.*, 2001).

Moreover, photochemical treatment employs oxidising agents (O_3 and H_2O_2) or heterogeneous catalysts (TiO_2, ZnO_2, Mn and Fe) in the presence or absence of an irradiation source leading to the generation of OH^- radicals and thus resulting in the destruction of dyes *(*Vandevivere *et al*., 1998; Forgacs *et al*., 2004; Anjaneyulu *et al.* , 2005; Wang *et al*., 2009). Sludge production and foul odour are greatly reduced in this method, whilst by-products like halides, metals, inorganic acids, organic aldehydes might be generated (Yang *et al*., 1998; Robinson *et al*., 2001).

A sorbent named cucurbituril been used in water treatment, e.g. for removing textile wastes from wastewater (Karcher *et al*., 2001b). Cucurbituril (a cyclic polymer of glycoluril and formaldehyde) exhibited a good sorption capacity of various textile dyes. A phenomenon like the formation of host-guest complexes with aromatic compounds, hydrophobic interaction, and formation of insoluble aggregates (cucurbituril-dye-cation) are the few proposed mechanisms of action of removal of dyes (Robinson *et al.*, 2001).

However, these physicochemical processes are expensive, less efficient, inapplicable towards a wide variety of dyes (Banat *et al.*, 1996) and require addition of chemical additives that are hazardous to the environment (Robinson *et al*., 2001). Another issue associated with these processes is a disposal problem due to the accumulation of concentrated sludge (Robinson *et al.*, 2001).

Additionally, several conventional methods exist for dephenolisation, problems like the formation of hazardous products, cost factor, inefficient removal and applicability to a limited concentration range are some of the drawbacks associated with them (Ryan *et al.*, 2007). Similarly, higher removal rates for antibiotics have been achieved by various AOP's such as photolysis, hydrogen peroxide combined with UV (UV/H_2O_2), ozonation, Fenton (H_2O_2/Fe^{2+}), photo fenton treatment ($H_2O_2/Fe^{2+}/UV$) and electrochemical processes (Reungoat *et al.*, 2012; Periša *et al*., 2013; Wu *et al.*, 2013).

Even though degradation of pollutant with physiochemical method(s) has been achieved, this does not always guarantee a reduction in the toxicity of contaminants such as antibiotics. In a study done by Zhu *et al.* (2016), the degradation products of fluoroquinolones (FQ's) by

electrochemical oxidation were found to be more toxic than the parent compound itself. Similar findings were achieved on the treatment of oxytetracycline via ozonation (Li *et al.*, 2008). The table given below discusses the relevant features, advantages, and disadvantages of each of the approaches **(Table 2.3).**

Table 2.3 Advantages and disadvantages of various physicochemical methods used to remove dyes from industrial effluents (Robinson *et al.*, 2001).

Physical/Chemical methods	**Advantages**	**Disadvantages**
Fenton's reagent	Effective decolourisation of both soluble and insoluble dyes	Sludge generation
Photochemical	No sludge production	Formation of by-products
Ozonation	Applied in the gaseous state: no alteration of volume	Short half-life (20 min)
NaOCl	Initiates and accelerates azo-bond cleavage	Release of aromatic amines
Cucurbituril	Good sorption capacity for various dyes	High cost
Electrochemical destruction	Breakdown compounds are non-hazardous	High cost of electricity
Activated carbon	Good removal of a wide variety of dyes	Very expensive
Peat	Good adsorbent due to cellular structure	Specific surface areas for adsorption are lower than activated carbon
Wood chips	Good sorption capacity for acid dyes	Requires long retention times
Silica gel	Effective for basic dye removal	Side reactions prevent commercial application
Membrane filtration	Removes all dye types	Concentrated sludge production
Ion exchange	Regeneration: no adsorbent loss	Not effective for all dyes
Irradiation	Effective oxidation at lab scale	Requires a lot of dissolved O_2
Electrokinetic coagulation	Economically feasible	High sludge production

Biological agents can offer efficient methods that are economic, environmentally safer and can address the limitations posed by existing conventional approaches to treat phenolic compounds, antibiotics, and dyes.

2.3.3 Biological methods

Biocatalysts (either whole cells or enzymes) can play a fundamental role in reducing the environmental footprint of the chemical processes. Biocatalysis follows the concept of green chemistry which is to develop or improve a wide range of processes that reduces the generation of toxic waste products that are hazardous to the environment and human health. In addition, this greener alternative to chemical processes also reduces the consumption of raw material and energy (Alcalde *et al.,* 2006).

In this respect, the use of microbes in bioremediation is an expanding area of biotechnology (Iwamoto and Nasu, 2001). Typically, bioremediation involves the use of microbes, plants or enzymes for removal of the contaminants from the environment (Rayu *et al*., 2012).

2.3.3.1 Use of microbes

Role of microbes has been explored in bioremediation of several environmental contaminants owing to their diversity, versatility and metabolic potential (Igbinosa *et al.*, 2013). Below we discuss a few advantages of using microbes in bioremediation:

Advantages:

1. This approach is a simple, straightforward and comparatively low investment approach.
2. It incorporates multiple oxidative enzymes that have a cooperative action that might be more effective than a single enzyme.

3. The property of biosorption is another factor which enhances the potential of whole cells in bioremediation. Furthermore, this property can be useful in bioremediation of compounds having a high octanol/water partition coefficient (Rubilar, *et al*., 2008; González *et al.*, 2010).

Limitations

1. The production of microbial cultures is costly and time-consuming.
2. Limited mobility of microbes in the soil renders them ineffective in treating contaminated soils and other solid material.
3. The presence of carbon sources might compete as a microbial nutrient with the toxic compounds.
4. Extremes of pH, temperature, toxins, predators, chemical shock, high concentration of pollutant might inactivate the microbial cells metabolically.
5. Maintenance of active cells is difficult during transportation.
6. Biodegradation via whole cells takes longer (usually several days).
7. Requires the addition of a co-substrate (such as glucose) in order to increase the degradation efficiency (Martínková *et al.*, 2016).
8. Effluents to be treated might not always support the growth of microorganisms (Kolhe *et al.*, 2015).
9. The efficiency of biodegradability and mineralisation is greatly dependent on characteristics of wastewaters such as temperature, pH, dissolved oxygen (DO), and an organic load which requires adjustment (Rana *et al.*, 2017).

2.3.3.2 Use of enzymes

Enzymes are natural catalysts that can be found in all living organisms. Enzyme technology offers new avenues for the clean-up of various contaminated sites where other methods have

proven to be inefficient (Ahuja et al., 2004). Standardising the optimum conditions, handling and storage of enzymes is simple and straightforward. Additionally, inexpensive crude enzyme extracts can successfully be applied in wastewater treatments (Kolhe *et al.*, 2015). The roles of several enzymes have been determined and have been employed in many remediation processes (Saitoh *et al.*, 2011). Enzymatic bioremediation is simpler and an attractive alternative than the whole organism. Enzymes have been used in degradation of several xenobiotics such as polycyclic aromatic hydrocarbons (PAHs) (Samanta *et al.*, 2002), pesticides (Torres-Duarte *et al.*, 2009), pulp bleaching (Bajpai, 2004), polynitrated aromatic compounds (Ramos *et al.*, 2005) and synthetic dyes (Soares *et al.*, 2001). From an environmental perspective, the use of enzymes offers several advantages over chemical processes or whole cells (Ahuja *et al.*, 2004), and these are discussed in detail below (Kolhe *et al*., 2015):

- Biotransformation includes a series of reactions catalysed by enzymes, where most of the adverse factors are alleviated and doesn't generate toxic products. In addition, enzymes are biodegradable as compared to the sludge formed due to biomass accumulation which is a matter of concern when using the whole cells.
- Bioavailability of enzymes can be enhanced by introducing organic solvents or surfactants, which are feasible.
- Enzymes can perform/function at mild conditions, thereby eliminating the use of harsh chemicals or extreme environments. This saves energy as well as prevents pollution.
- Using recombinant DNA technology and immobilisation procedures, enzymes can be produced at a larger scale and tailored according to the requirements and can be rendered more stable and reused in continuous processes.

- Enzymes are specific in nature, resulting in fewer side by-products in the process of production.
- In contrast to whole cells, enzymes are not inhibited by-products (or inhibitors) of microbial metabolism (Kolhe *et al.*, 2015).

However, it is essential to find robust enzymes which operate under an array of conditions, with higher substrate affinity (K_m in the micromolar range) and low dependency on expensive redox cofactors which might hinder their use in a commercial setting (Alcalde *et al.*, 2006).

In this respect, laccases have shown to be significant in transforming toxic compounds to insoluble complexes via oxidative enzyme coupling. In addition to the removal of contaminants, detoxification of treated effluents has also been reported (Dwivedi *et al.*, 2011), thereby making laccases an ideal biocatalyst.

2.3.3.3 Laccases as ideal biocatalysts for bioremediation

Laccases are ideal biocatalysts due to their ability to use oxygen as an electron acceptor and its broad substrate specificity (Wang *et al.*, 2015). Inclusion of redox mediators could further expand the substrate spectrum (Hilde *et al.*, 2009). Laccases have many possible applications in bioremediation. Laccases were found to successfully remove herbicide isoxaflutole (Mougin *et al.*, 2000), isoproturon (Zeng *et al.*, 2017), remazol brilliant blue SN4R (Hou *et al*., 2004), trace organic contaminants (TrOC) (Nguyen *et al.*, 2014), oxidation of polycyclic aromatic hydrocarbons (PAH) (Majcherczyk *et al.*, 1998) and transformation of the fungicide cyprodinil (Kang et al., 2002), and carbamazepine (Naghdi *et al*., 2018). Laccase-mediator system (LMS) has been extensively studied in the oxidation of recalcitrant PAHs which are the main components of several ship spills. In this sense, LMS is being included in several enzymatic

bioremediation programs (Alcalde *et al.*, 2006). **Table 2.4** depicts the degradation of phenolic compounds by several laccases.

Table 2.4 Biodegradation of phenols and its derivatives by laccases

Compound	Enzyme source	Conversion (%)	References
2 Chlorophenol (2-CP)	*Coriolus versicolor*	98.6	Kadhim *et al.*, 1999
2-CP	*T. versicolor*	43	Dec and Bollag, 1994
2,4-DCP	*C. versicolor*	92.3	Kadhim *et al.*, 1999
2,4,6-TCP	*C. versicolor*	100	Kadhim et *al.*, 1999
2,4-DCP	*C. versicolor*	50	Zhang *et al.*, 2009
Brominated aromatics	*T. versicolor*	65–85	Uhnáková *et al.*, 2009

In several reports, it was found that laccase can be inactivated while converting phenolic compounds due to the interaction of oxidative polymerisation products with the enzyme (Kulys *et al*., 2003 Nicell, 2003; Kim and Nicell, 2006). In some reports, addition of a surfactant in the reaction mixture was found to protect the enzyme from inactivation by reducing the interaction between the polymerised products with the enzyme (Zhang *et al.*, 2012). The stability of an enzyme could be the result of surfactant induced changes in the enzyme's conformation or its active site (Zhang *et al.*, 2012).

However, in addition to these problems, other limitations of laccases are further discussed in full detail below.

2.4 Limitations of present laccase applications

Current applications are basically focused on fungal laccases due to the extensive amount of research that has been done on fungal laccases. Contrary to their bacterial counterparts, fungal

laccases are well characterised, possess high redox potential, and have high catalytic efficiency towards a wide range of substrates. Fungal laccases are highly glycosylated enzymes and therefore can't be expressed in prokaryotic hosts. To date, there is only a single report where a fungal laccase from *Cyathus bulleri* has been expressed in *E.coli* (Garg *et al.*, 2008). This makes the use of eukaryotic expression system mandatory. However, the cultivation and genetic manipulations are a complicated task thereby making production and optimisation of fungal laccases time-consuming.

White rot fungi have been known to secrete different types of oxidative enzymes such as lignin peroxidases (LiPs), manganese peroxidases (MnPs), peroxidases and laccases (Huang *et al.*, 2013). However, fungal laccases are found to be active in acidic reaction conditions (Zapun *et al.*, 1995)**.** Therefore, fungal laccases have limited applications at neutral and alkaline pH values such as in the treatment of wastewaters, in bleaching of paper pulp, implanted biofuel cells or in washing. However, various researchers have tried to widen the pH-activity profile of fungal laccases (Torres-Salas *et al.*, 2013; Yin *et al.*, 2019). In addition, fungal enzymes can be costly to produce and difficult to optimise by protein engineering (Huang *et al.*, 2013). Poor availability of genetic sequence information, glycosylation and intron processing are other limitations associated with heterologous expression of fungal laccases (Santhanam *et al.*, 2011). Therefore, bacterial laccases are now also being investigated as potential candidates for industrial application.

2.5 Bacterial laccases

A distinct feature of bacterial laccases is that they consist of only two domains as compared to the three domains of the fungal laccases (Singh *et al.*, 2017). Bacterial laccases are comparatively more robust owing to their thermal and pH stability, inexpensive production and

are amenable for protein engineering (Huang *et al.*, 2013). Several studies have reported the ability of bacterial laccases to oxidise a wide range of phenolic compounds, whilst the bulky non-phenolic units restricted its action on lignin polymer (Bugg *et al.*, 2011b). However, with the aid of mediators such as ABTS and 1, hydroxybenzotriazole (HBT), bacterial laccases could depolymerise lignin (Moilanen *et al.*, 2014). Apart from the role of bacterial laccases in lignin degradation, features such as broad substrate specificity permit laccase to be used in targeting various recalcitrant compounds (Castanera *et al.*, 2012) or modification of lignocellulosics in an eco-friendly manner (Bugg *et al*., 2011a). Bacterial laccases are not well studied or characterised as compared to their fungal counterparts (Bugg *et al.*, 2011b). However, bioinformatics analysis demonstrates the wide distribution of laccases or laccase-like enzymes in bacteria (Ausec *et al.*, 2011). Though physiological functions of most characterised bacterial laccases remain unknown. However, in a few cases, physiological roles have been described such as spore pigmentation for CotA from *B. subtilis* (Hullo *et al.*, 2001), copper homeostatis for CueO from *E.coli* (Grass and Rensing, 2001; Outten *et al.*, 2001) and CopO from *Corynebacterium glutamicum* (Schelder *et al.*, 2011)**.** Until now, the characteristics and biotechnological application of these enzymes are poorly investigated and very few reports on bacterial laccases have been published reporting the thermal stability and alkaline activity profiles as compared to their fungal counterparts (Machczynski *et al.*, 2004; Ruijssenaars and Hartmans, 2004; Miyazaki, 2005). For example, the laccase from *Thermus thermophilus* exhibited extreme stability at high temperatures with a half-life of thermal inactivation at 80°C of more than 14 h (Miyazaki, 2005) while laccases from *Bacillus halodurans* and *Streptomyces coelicolor* exhibited maximum activities towards syringaldazine or 2,6-dimethoxyphenol at pH values of 7.5 or 9.4 (Machczynski *et al.*, 2004; Ruijssenaars and Hartmans, 2004). Such properties might help to minimise the limitations of fungal laccases

such as in extending the range of feasible reaction conditions (towards higher pH values, elevated reaction temperatures and prolonged production processes).

Moreover, Gunne & Urlacher, (2012) characterised an alkali laccase Ssl1 (from *Streptomyces sviceus*) which is a small laccase (32.5 kDa) consisting of only two cupredoxin like domains and forms trimers in solution. They discovered it by exploiting the Laccase engineering database (LccED) derived from genome mining and it was found to oxidise ABTS 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and phenolic substrates such as guiacol, syringaldazine and 2, 6-dimethoxy phenol. The optimal pH for oxidation reactions was found to be substrate dependent: for phenolic substrates, the highest activities were detected at alkaline conditions (pH 9.0 for 2, 6-dimethoxyphenol (DMP) and guaiacol and pH 8.0 for syringaladazine), while the highest reaction rates with ABTS were observed at 4.0. Ssl1 demonstrated a remarkable stability at a higher temperature ($T_{1/2}$, 60 °C = 88 min) and in a wide pH range (pH 5.0 to 11.0), despite it being derived from mesophilic organism. Apart from this, it also exhibited stability against detergents and organic solvents. In another study, a small laccase called SLAC (from *Streptomyces coelicolor*) was characterised and it consisted of only two domains; it lacks the second domain yet exhibits good activity. SLAC (approximately 32 kDa molecular mass) was found to be resistant to detergents, possessed high thermal stability, and was active as a dimer in gels and solutions. It was also found to be active against several substrates. SLAC activity against 2, 6-dimethoxyphenol reached a maximum at an unexpectedly high pH of 9.4, thereby proposing its suitability for application at alkaline pH (Machczynski *et al.*, 2004). Various studies have shown that laccases from bacilli showed activity at neutral to alkaline pH (Koschorreck *et al*., 2008; Zhang *et al*., 2013). In general, bacterial laccases were found to exhibit thermal stability, while fungal laccases were found to be more tolerant towards organic solvents, high salt concentrations and common inhibitors.

However, low redox potential as well as narrow range of substrates are some of the drawbacks which limit their use in industrial applications (Singh *et al*., 2011).

Although bacterial laccases can be expressed in prokaryotic expression systems such as *E.coli*, their heterologous complement mostly results in enzyme which do not contain a full complement of copper ions as illustrated in several reports (Enguita et al., 2003; Koschorreck et al., 2008; Gunne and Urlacher, 2013). As a result, activity yields of bacterial laccase in *E. coli* were unsatisfactory. SLAC'S stability over a wide pH and temperature range, ease of production and engineering, make it a potentially attractive catalyst for a wide range of applications (Singh *et al*., 2017). However, large scale applications are marred by large consumption and difficulty to reuse in multiple catalytic reactions (Fernanades *et al*., 2017; Ranjan *et al.*, 2017). Bacterial laccases could present a source of low-cost enzymes as they can be readily expressed in recombinant host and are amenable to protein engineering (Santhanam *et al*., 2011). However, low expression yields remain one of the major obstacles in their applicability in industrial applications. **Table 2.5** refers to the list of several bacterial laccaaes expressed in heterologous hosts.

Table 2.5 List of heterologously expressed bacterial laccases

Laccase	Source	Host	Reference
SLAC	*Streptomyces coelicolor*	*E.coli*	Machczynski *et al.*, 2004
CotA	*Bacillus subtilis*	*E.coli*	Martins *et al.*, 2002
CotA	*B. subtilis*	*P. pastoris*	Fan *et al.*, 2015
CotA	*B. subtilis*	*P. pastoris*	Wang *et al.*, 2015
EpoA	*Streptomyces griseus*	*E.coli*	Endo *et al.*, 2003
non-blue/spore laccase	*Bacillus amyloliquefaciens*	*P. pastoris*	Chen *et al.*, 2015
polyphenoloxidase (PPO)	*Streptomyces lavendulae* REN-7	*E.coli*	Suzuki *et al.*, 2003
CueO	*E.coli*	*E.coli*	Grass and Rensing, 2001
Thermophilic bacterium (TTC1370)	*Thermus thermophilus* HB27	*E.coli*	Miyazaki, 2005
bh2082	*Bacillus halodurans* C-125	*E.coli*	Ruijssenaars and Hartmans, 2004
Ssl1	*Streptomyces sviceus*	*E.coli*	Gunne and Urlacher, 2012
Lac15	marine microbial metagenome	*E.coli*	Fang *et al.*, 2011
spore laccase	*Bacillus licheniformis* LS04	*E.coli*	Lu *et al.*, 2012
SilA	*Streptomyces ipomoea* CECT 3341	*E.coli*	Molina-Guijarro *et al.*, 2009
MnxG	*Bacillus* species	*E.coli*	Dick *et al.*, 2008
Solvent, Metal And Surfactant-Stable Extracellular Bacterial Laccase	*Proteus vulgaris* ATCC 6896	*Proteus vulgaris* ATCC 6896	Britos *et al.*, 2018
LMCOs	*Streptomyces* sp. C1	*Streptomyces sp.* C1	Lu *et al.*, 2013

2.6 Strategies to make bacterial laccases viable catalysts

In order to make bacterial laccases viable biocatalysts, several strategies can be explored such as protein engineering, expression in a recombinant host, use of dual promoters, immobilisation and the inclusion of mediators to expand laccase catalysis.

2.6.1 Laccase mediator systems

Laccases have lower redox potential (≤0.8 V) as compared to ligninolytic peroxidase (>1 V). Therefore, the oxidation action of laccases would be restricted only to those substrates within the laccase redox potential range. For instance, when laccase is used in the treatment of pulp mills wastewaters, only phenolic lignin, which constitutes less than 20% of lignin polymer, will be oxidisable while non-phenolic moieties (redox potential of above 1.3 V) can't be directly oxidised by laccases (Li *et al.*, 2010). However, this limitation can be overcome by the usage of redox mediators which are low molecular weight laccase substrates acting as "electron shuttles" between the recalcitrant compound (such as non-phenolic lignin units) and the enzyme (Nguyen *et al.*, 2016). Few examples on use of laccase-mediator systems for various applications are given in **Table 2.6**. Mediators also oxidise complex substrates that don't enter the enzyme's active site due to steric hindrances. After oxidation by the enzyme, mediators diffuse away from the enzyme pocket and enable the oxidation of the complex substrates which in principle are not laccase substrates (Bourbonnais & Paice, 1990). Use of mediators extend the range of pollutants that can be targeted for treatment by laccases (Kim and Nicell, 2006). By using mediators, laccases have been applied to decrease the kappa number during pulp delignification (Ibarra *et al.*, 2006; Moldes *et al.*, 2008). Mediator-based laccase catalysis has been exploited in a wide range of applications including degradation of dyes, herbicides, pesticides, organic pollutants polycyclic aromatic hydrocarbons, and biosensor or biofuel cell development. In the pulp and paper industry, there is an increased attention towards novel

laccase-mediator based bleaching technologies due to the adverse environmental impact of currently used chlorine based oxidants in delignification (Xu *et al.*, 2000).

Despite the proven efficacy of certain artificial mediators (ABTS, 1-hydroxybenzotriazole (HBT), violuric acid, TEMPO) in degradation of recalcitrant compounds, excessive costs and the possible generation of toxic species (leading to biocatalyst inactivation) precludes their application on an industrial scale (Astolfi *et al.*, 2005). However, there are several reports, where certain natural phenolic compounds obtained from lignin polymers are capable of mediating oxidation of non- phenolic compounds with comparable or improved performance than the artificial mediators (Johannes and Majcherczyk, 2000; Astolfi *et al.*, 2005; Camarero *et al.*, 2005; Calcaterra *et al.*, 2008; Nousiainen *et al.*, 2009; Cañas and Camarero, 2010). These naturally occurring phenolic mediators can be procured from lignocellulosic, effluent streams or as by-products from plant manufacturing processes offering an economical and eco-friendly approach as they can be easily extracted from the source at a low cost (Gutiérrez *et al.*, 2007).

Table 2.6 List of selected reports using the laccase-mediator systems in various applications

Name of the mediator	Application	Reference
ARTIFICIAL		
ABTS	Oxidation of organic dyes	(Solís-Oba *et al.*, 2005)
	Production of active and decolorized chitosan-genipin films	(Gonçalves *et al*., 2017)
	Biotransformation of carbamazepine	(Naghdi *et al.*, 2018)
	Depolymerisation of kraft lignin	(Bourbonnais *et al.*, 1995)
	Delignification of Kraft pulp	(Bourbonnais and Paice, 1996)
	Oxidation and coupling of Guaiacylglycerol-β-guaiacyl ether (GBG)	(Hilgers *et al.*, 2018)
	Decolourisation of synthetic dyes	(Wong and Yu, 1999)
	Removal of pentachlorophenol (PCP)	(Jeon *et al.*, 2008)
	Decolorisation of anthraquinone dye Remazol Brilliant Blue SN4R	(Hou *et al*., 2004)
HBT	Decolorisation of synthetic dyes	(König, 2002)
	Bleaching of eucalyptus kraft pulp	(Moldes and Vidal, 2008)
	Delignification of kraft pulp	(Bourbonnais *et al*., 1997)
	Bleaching eucalypt kraft pulp	(Ibarra *et al.*, 2006)
	Removal of trace organic contaminants (TrOC)	(Nguyen *et al.*, 2014)
	Degradation of the herbicide isoproturon	(Zeng *et al*., 2017)
	Oxidation of Polycyclic Aromatic Hydrocarbons (PAH)	(Majcherczyk *et al*., 1998)
	delignification and sterol removal from eucalypt kraft pulps	(Speranza *et al.*, 2007)
ABTS, HBT*	bleaching of non-wood high-quality paper pulp	(Camarero *et al.*, 2004)
ABTS, HBT*	Decolourisation of Sella Solid Red (a disazo dye) and Luganil Green (a copper phthalocyanine dye)	(Rodríguez Couto *et al*., 2005)
(ABTS), (HBT), *N*-hydroxyacetanilide (NHA), polioxometalates, violuric acid (VA) and (2,2,6,6- tetramethylpiperidin-1- yloxy) (TEMPO))	reactive textile dyes degradation	(Tavares *et al.*, 2008)
HBT and violuric acid *	oxidation of a nonphenolic lignin dimer, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propan-1,3-diol (I)	(Li *et al.*, 1999)
TEMPO	Biotransformation of ritalinic acid	(Kobakhidze *et al.*, 2018)
	oxidation of natural glycosides	(Baratto *et al.*, 2006)
	oxidation of alcohols to carbonyl compounds	(Fabbrini *et al.*, 2001)
violuric acid	Decolorization of Remazol Brilliant Blue R (RBBR)	(Soares *et al.*, 2001)
NATURAL		
4-hydroxybenzoic acid, and 4-hydroxybenzyl alcohol	oxidation of acenaphthene, acenaphthylene, anthracene, and fluorene (PAH's)	(Johannes and Majcherczyk, 2000)
syringaldehyde	Oxidation of Methoxylated Benzyl Alcohols	(Kawai *et al*., 1989)
Natural phenolic compounds (hydroquinone, catechol, o-methoxyphenol, m-methoxyphenol, p-methoxyphenol, 2,6-dimethoxyphenol, *syringaldehyde, syringic acid, protocatechuic acid, p-coumaric acid, ferulic acid, vanillic acid, vanillin)	Transformation of the fungicide cyprodinil	(Kang *et al.*, 2002)
syringaldehyde, acetosyringone, 2,6-dimethylphenol, 2,4,6-trimethylphenol, ethyl vanillin, acetovanillone, vanillin, vanillyl alcohol and *p*-coumaric acid	Paper pulp delignification	(Camarero *et al.*, 2007)
*syringaldehyde (SA), *acetosyringone (AS) and *p*-coumaric acid (PCA)	biobleaching of flax pulp	(Fillat *et al*., 2010)
4-hydroxybenzoic acid, syringic acid, vanillic acid, *p*-coumaric acid, ferulic acid, sinapinic acid	Transformation of chlorophenols	(Itoh *et al*., 2000)
methyl syringate	Fibre modificataion of kraft pulp	(Liu *et al.*, 2009)
4-hydroxybenzoic acid	Degradation and reduction of toxicity of fungicide imazalil	(Maruyama *et al.*, 2007)
syringaldehyde	Transformation of triclosan	(Murugesan *et al.*, 2010)
syringaldehyde	Transformation of malachite green	(Murugesan *et al.*, 2009)
syringaldehyde* and acetosyringone*	Halogenated pesticide transformation	(Torres-Duarte *et al*., 2009)

*most effective out of a series of mediators used in the study

2.6.2 Enzyme Immobilisation

Laccase is a versatile biocatalyst, due to its high activity, specificity, selectivity and its ability to perform complex processes in experimental and environmental conditions (Mateo *et al.*, 2007). However, the use of free enzymes has certain practical limitations such as low stability, high production costs (Hu *et al*., 2007; Rekuć *et al.*, 2009), non-reusability and sensitivity towards denaturing agents (Durán *et al.*, 2002). Metal ions, salts, detergents, chelators and other components can severely affect the enzyme catalysis, thereby questioning the applicability of free enzymes at a large scale. High production costs (Hu *et al*., 2007; Rekuć *et al.*, 2009) and non-reusability (Durán *et al.*, 2002) are other limitations associated with the usage of free enzymes.

Enzymes as decontaminating agents in environmental clean-up have gained popularity as no side reactions are involved in contrary to other methods. However, low stability and inactivation of enzymes are major hindrances in its applicability in the practical application (Hu *et al*., 2007). Immobilisation is an effective technique as it offers several advantages such as thermo-stability and resistance to extreme conditions (Georgieva *et al.*, 2008; Arica *et al.*, 2009). Enzyme immobilisation shifts enzyme's properties such as optimum pH or temperature, and kinetic parameters, as well as strengthening the enzyme's structure (Rekuć *et al.*, 2009). Laccase immobilisation has been attempted using different carriers, like oxirane acrylic beads, magnetic chitosan microspheres, activated carbon, etc. (Hu *et al*., 2007). However, to the best of our knowledge studies on immobilisation of small laccases from *Streptomyces coelicolor* have never been reported.

Despite the availability of solid support matrices, exploitation of novel immobilisation matrices has been increasingly gaining attention (Bilal *et al.*, 2018). Among several solid matrices,

nanoparticles (NP's) are promising candidates for immobilization owing to their low toxicity and unique physio-chemical properties. However, it is essential to functionalise the surface of the nanoparticle to enable surface stability and biocompatibility (Poorakbar *et al.*, 2018).

Furthermore, the efficiency of nanostructured materials in immobilisation of enzymes has been demonstrated recently. Materials such as nanoparticles, nanocomposites, nanotubes, nanofibers, nanoporous media and graphene possess large surface areas which increase loading of enzyme thereby facilitating the kinetics of the reaction, as well as lower mass transfer resistance which determines the efficiency of the biocatalyst (Puri *et al.*, 2013). The most common nanomaterials used for enzyme immobilisation include carbon nanotubes and magnetic nanoparticles.

2.6.2.1 Carbon nanotubes

Carbon nanotubes (CNTs), owing to their unique physiochemical and electrical properties, have received considerable attention (Hu *et al.*, 2009). They are super adsorbents (in comparison to activated carbon) and possess hollow structure, large surface area, light mass density and their strong interaction with molecules makes them applicable in removing various hazardous materials from aqueous solutions (Ren *et al.*, 2011). CNTs include single-walled carbon nanotubes (SWCNTs), multi-walled carbon nanotubes (MWCNTs), graphene, single-walled carbon nanohorns (SWNHs, identical to SWCNTs but closed by a cone at one end), fullerene and nanodiamonds (NDs) **(Fig. 2.7)** (Liu and Liang, 2012). Usage of carbon nanotubes at a large scale can be limited by their dispersive nature posing process engineering difficulties (Ai and Jiang, 2010).

2.6.2.2 Magnetic Nanoparticles

Magnetic nanoparticles (MNP) offer exceptional properties of prolonged mechanical stability, easy recovery from reaction mixture, as well as reusability by simple usage of a magnetic field. These features can greatly reduce the operational costs (Vinoth and Cabana, 2016).

However, combining the carbon materials with magnetic nanoparticles can offer the possibility of a creation of a robust hybrid composites possessing synergistic properties (Pereira *et al.*, 2017) of adsorptivity and magnetic separability (Ai and Jiang, 2010). Several studies exploited the combination of both materials (CNT@MNP composites) and achieved success in the removal of various contaminants (Oliveira *et al.*, 2002; Ai *et al.*, 2010; Gupta *et al.*, 2011). The hybrid composites can be easily recovered by applying the magnetic field via a magnet (Huang *et al.*, 2015).

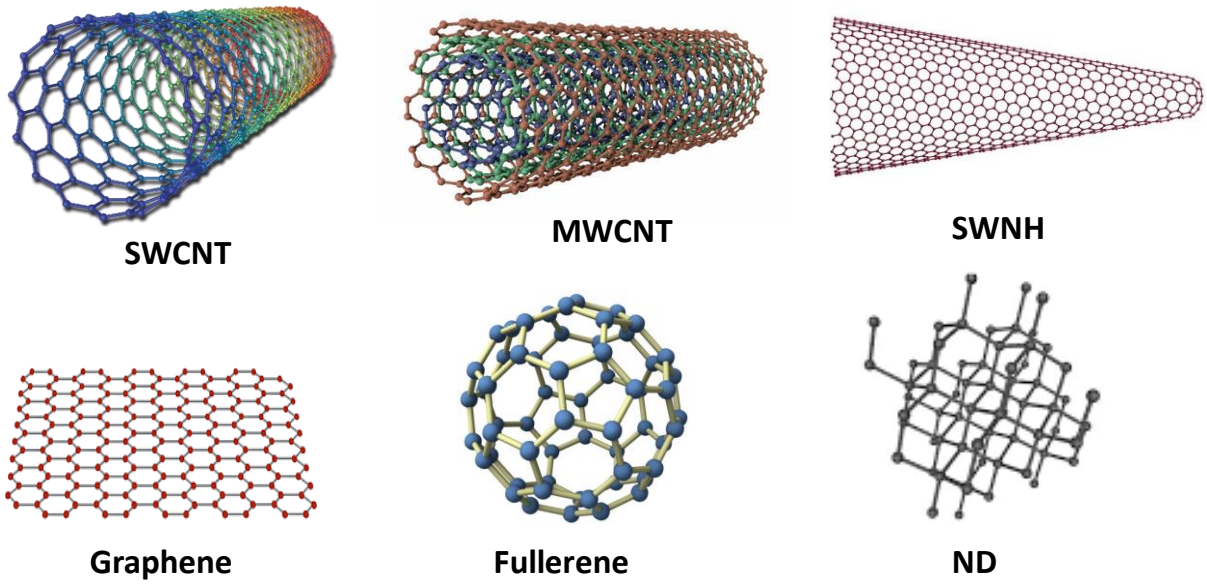


Fig. 2.7 Schematic representation of different carbon nanomaterials **(**Kanoun *et al.*, 2014 with modifications)

2.6.3 Protein engineering

Biological catalysts have gained attention due to their specificities, selectivities and activities. Characteristics of biocatalysts such as chemo-, enantio- and regioselectivities are desirable in the development of fine chemicals and pharmaceuticals. However, these natural biocatalysts that support the chemical reactions in various living organisms are often not optimal for their use in industrial processes (Damián-Almazo and Saab-Rincón, 2013). Identification of new biocatalysts (for example, by screening of soil samples or strain collections by enrichment cultures) does not always yield suitable enzymes for a given synthetic problem. Since the late 1990s, enzyme engineering has been most profound and interesting transformation (Georgiou *et al.*, 1999). Protein engineering is a powerful tool for improving enzyme properties. Enzymes with desirable properties such as high thermostability, enhanced activity and high specificity under industrial conditions can be obtained by optimising process conditions and by protein engineering (Singh *et al.*, 2013). **Fig. 2.8** shows different protein engineering strategies that are currently employed in research. However, In general, a protein is engineered by changing its amino acid sequence at the nucleotide level either by genetic technique or by chemical method. Hereafter, the variant protein generated can then be tested for changed properties. Many desirable changes in terms of expression level, substrate specificity, thermal stability, tolerance towards extreme pH values, metal ions and organic solvents can be brought by protein engineering (Damián-Almazo and Saab-Rincón, 2013).

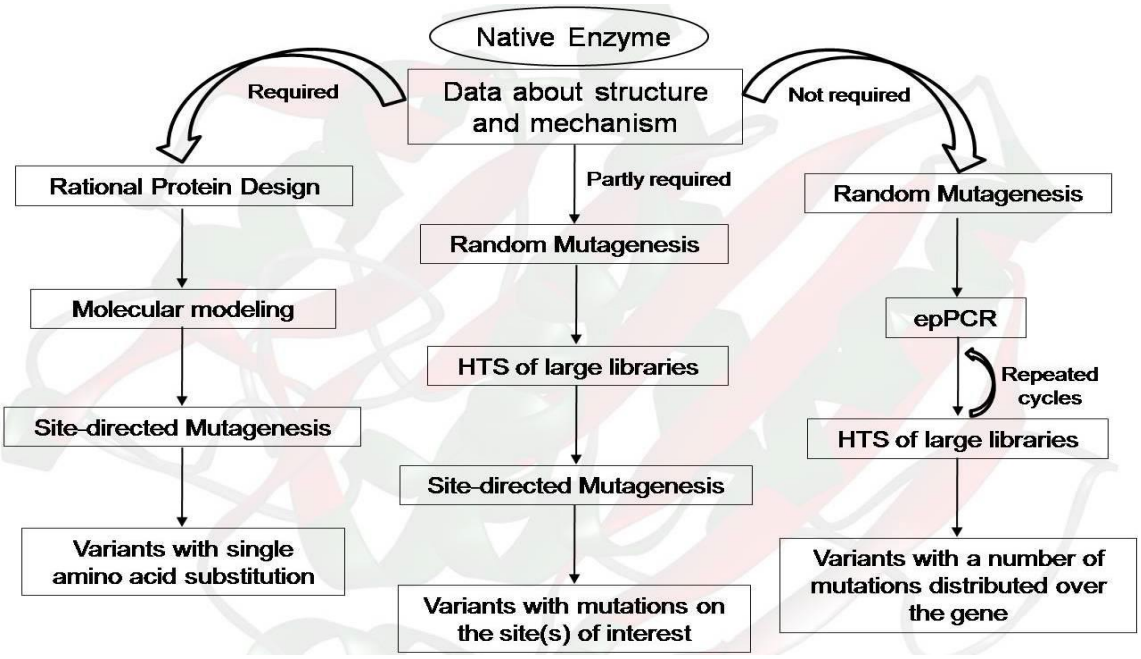


Fig. 2.8 Schematic representation of protein engineering strategies (Singh *et al.*, 2013).

Broadly, protein engineering can be divided into two categories: rational approach and directed evolution. However, it is common to combine these two approaches (Damián-Almazo and Saab-Rincón, 2013). Even though both approaches have achieved great success, each has its own limitations. The choice of method depends upon case to case, such as the property of interest, availability of structural and mechanistic knowledge as well as practical considerations like the availability of a high throughput screening system.

2.6.3.1 Rational design: Here, specific residues for site-directed mutagenesis are selected based on prior knowledge of the structure-function relationships (Mate and Alcalde, 2015).

2.6.3.2 Directed evolution: is based on Darwinian principles of natural evolution and does not require structural knowledge of the protein (Mate and Alcalde, 2015). However, it does require remarkable screening efforts in order to analyse thousands of clones. The availability

of reliable screening efforts for shortlisting the desired mutant from the rest of the clones is a major bottleneck for this approach. So far, calorimetric screening has been the only high throughput screening method for the directed evolution of laccases (Alcalde and Bulter, 2003; Pardo *et al.*, 2013).

2.6.4 Recombinant protein expression

Laccase production from native microorganisms is very low (Ranjan and Satyanarayana, 2016) which is insufficient to satisfy the market demands. Thus, reducing the cost of production and improving the production levels remains the major goal.

2.6.4.1 *Escherichia coli*: Extensive research has been focussed on the search for simple heterologous expression systems. *E.coli* is the most sought after expression host (Santhanam *et al.*, 2011). However, the major limitation lying with overexpression of recombinant proteins in *E.coli* is the formation of inclusion bodies (Fang *et al.*, 2014; Rosano and Ceccarelli, 2014), which leads to low production yield of soluble protein (Martins *et al.*, 2002; Suzuki *et al.*, 2003; Fang *et al.*, 2011; Chen *et al.*, 2015). Furthermore, difficult purification procedures of inclusion bodies further contribute to the production costs (Santhanam *et al.*, 2011).

2.6.4.2 *Pichia pastoris*: *Pichia pastoris*, a methylotrophic yeast is an established expression host to produce biopharmaceuticals and industrial enzymes (Ahmad *et al.*, 2014). It has acquired a GRAS (Generally regarded as safe) status and has even been used in the production of several biopharmaceuticals (Fickers, 2014), approved for human use by the Food and Drug Administration (FDA) of USA [http://www.pichia.com] (Parashar and Satyanarayana, 2016). The popularity of this versatile system over other eukaryotic and

prokaryotic system can be attributed to its ability to grow at high cell densities, capability to perform post-translational modifications (such as correct protein folding, glycosylation, disulphide bridge formation), generates stable lines as linearised foreign DNA gets into the yeast chromosome via homologous recombination, high secretion efficiency, ease of genetic manipulation due to the well-characterised yeast expression vectors (Parashar and Satyanarayana, 2016; Yang and Zhang, 2017). Furthermore, the ability of *P. pastoris* for extracellular expression can serve as an alternative in the expression of foreign proteins as it greatly simplifies the purification steps. However, the long induction phase and low specific productivity are some of the drawbacks associated with the utility of *P. pastoris* in industries. Attempts such as the development of new promoters, codon optimisation, co-transformation of helper factors and generation of multicopy strains have been attempted by various researchers (Parashar and Satyanarayana, 2016).

2.6.5 Dual Promoter system in *Pichia pastoris*

The use of dual promoter system in *P. pastoris* have paved way to enhance the production level of target protein by increasing the transcription capacity of the promoter(s). Each promoter has certain specific requirements relating to its expression conditions. To achieve the optimal potential of dual promoter systems, it is crucial to balance the requirements of both the promoters involved. In addition, development and optimisation of fermentation conditions is another crucial factor to be considered (Öztürk *et al*., 2017). Double promoter systems have been used to enhance the transcription level of the cloned gene by appointing two promoters in expressing various enzymes (**Table 2.7**).

Table 2.7 Reports relating to the use of double promoters in *Pichia pastoris*

Reference	Gene expressed	Strain used	Plasmid	Promoter (s)	Regulation	Increase in production level
Waterham *et al.*, 1997	Wild-type and mutant AOX	GS115	pHIL-AI + pHIL-AI	P_{AOX1}	Inducible + Inducible	No comparison between single and double promoters
Wu *et al.*, 2003	Human granulocyte macrophage	GS115	pPIC9K + pGAPZαA	P_{GAP} + P_{AOX1}	Constitutive + Inducible	2-fold (P_{GAP})
He *et al.*, 2008	Thermostable alkaline β-mannanase	GS115	pGAPZαA + pPIC9	P_{GAP} + P_{AOX1}	Constitutive + Inducible	1.4-fold (P_{AOX1}) * 5.8-fold (P_{GAP})
Li *et al*., 2011	α-Amylase *	GS115	pGAPZC + pPIC9K	P_{GAP} + P_{AOX1}	Constitutive + Inducible	3–5% (P_{AOX1}) * 3–5% (P_{GAP})
Fang *et al.*, 2014	Thermostable lipase	GS115	pPIC9 K + pPICZαA, pFZα or pGAPZαA	P_{AOX1} + P_{FLD1}, P_{AOX1} or P_{GAP}	Inducible + Inducible or Constitutive	*P_{AOX1} + P_{GAP}: 1.10-fold (P_{AOX1}) P_{AOX1} + P_{FLD1}: 1.38-fold (P_{AOX1}) *P_{AOX1} + P_{AOX1}: 1.52-fold (P_{AOX1})
Tang *et al.*, 2014	Acidophilic β-mannanase	GS115	pPIC9K + pPICZαA	P_{AOX1} + P_{AOX1}	Inducible + Inducible	1.43-fold (P_{AOX1})
He *et al.*, 2015	Lipase	GS115	pGAPZαA + pPIC9K	P_{GAP} + P_{AOX1}	Constitutive + Inducible	* 5.6-fold (P_{AOX1}) * 7-fold (P_{GAP})
Parashar and Satyanarayana, 2016	Acidic α-amylase	X-33	pPICZαA + pGAPK αA	P_{GAP} + P_{AOX1}	Constitutive + Inducible	* 1.8-fold (P_{AOX1})
Parashar and Satyanarayana, 2016	Acid stable and thermostable phytase	X-33	pPICZαA + pGAPK αA	P_{GAP} + P_{AOX1}	Constitutive + Inducible	* 1.3-fold (P_{AOX1})

* denotes the increase in the level of production as compared to the control(s) used in the study (given in brackets)

Lipase from *Rhizomucor miehei*, expressed in *P. pastoris* dual promoter system produced up to six to seven-fold higher lipase activity (140 U/ml) as compared to a sole inducible promoter (25 U/ml) and constitutive promoter (20 U/ml). The lipolytic activity of the dual promoter harbouring strain was further enhanced to 175 U/ml after optimisation of culture conditions at the shake flask level (He *et al.*, 2015). In another study, heterologous production level of a thermostable lipase from *Thermomyces lanuginosus* was enhanced from 4350 U/ml (pPIC9K vector) to 4800, 6000 and 6600 U/ml when second expression vector (P_{GAP}-, P_{FLD1}- or P_{AOX1}-) was integrated respectively (Fang *et al*., 2014). In addition, the expression values were found to be directly proportional to the copy number. As assessed by the real-time quantitative PCR, copy numbers in dual strains were three, five and seven for 9K/GAP, 9K/FLD and 9K/AOX respectively as compared to the two copies in pPIC9K (Fang *et al.*, 2014). A recombinant *Pichia pastoris* strain expressing an acidophilic β-mannanase was constructed by cloning *Aspergillus usamii* YL-01-78 β-mannanase gene in pPIC9K and pPICZαA vectors separately. The highest recombinant β-mannanase activity obtained with single promoter system (pPIC9K) was 54.6 U/ml and rose to about 78.1 U/ml and 162.8 U/ml with dual promoter system and with the optimisation of the fermentation conditions (Tang *et al*., 2014). Likewise, combined usage of constitutive and inducible promoters (GAP and AOX1 promoters) enhanced the production level of thermostable alkaline β- mannanase from *Bacillus* sp. N16–5 upto 32.2 U/ ml as compared to the activity level of only 23.7 U/ml and 5.6 U/ ml from a single promoter system of P_{AOX1} and P_{GAP} respectively (He *et al.*, 2008). Wu *et al*. (2003) constructed a dual promoter system by consecutive transfection of *Pichia pastoris*, GS115 strain with P_{GAP} controlled human granulocyte-macrophage colony-stimulating factor ((hGMCSF) and P_{AOX1} controlled hGM-CSF to constitutively and inducibly express hGM-CSF. Upon methanol induction alone, the dual promoter system led to a two-fold increase in hGM-CSF expression level than the constitutively expressed hGM-CSF (pGAP- hGMCSF). In

another study, cloning of two genes (acidic α-amylase and phytase) from *Sporotrichum thermophile* under dual promoter led to 1.8 and 1.3-fold improvement in α-amylase and phytase production as compared with the single promoter (P_{AOX1}-based) system. A direct correlation of gene copy numbers with the production level was observed. The recombinant strain Phy-GAP-AOX and Amy-GAP-AOX integrated five and three copies of phytase and amylase gene as compared to the three under Phy-AOX and one copy under Amy-AOX (Parashar and Satyanarayana, 2016). On the contrary, in an unsuccessful attempt to produce α-amylase of *Rhizopus oryzae* under combined control of P_{AOX1} and $P_{GAP,}$ only 3-5% higher α-amylase activities was achieved as compared to that of the parent recombinant strain (Li *et al*., 2011).

2.7 Scope of the study

The role of laccases in various applications has been gaining research interest in recent years. Fungal laccases have predominantly been investigated for biotechnological applications. However, limitations associated with fungal laccases have prompted researchers to consider bacterial laccases as alternatives. Most bacterial laccases can tolerate a wide range of pH, temperature and metal ions, and therefore have been gaining popularity. However, poor enzyme yields and catalytic properties are some of the limitations associated with bacterial laccases.

In view of these considerations, the SLAC from *Streptomyces coelicolor* A3(2) was selected as a potential candidate for the investigation of strategies for improving production levels and catalytic properties. In addition, the recombinant SLAC was explored for application in bioremediation and surface functionalisation.

CHAPTER 3

Improving the catalytic properties of small laccase from *Streptomyces coelicolor* A3(2) via site-directed mutagenesis

Abstract

Small laccases can be a potential industrial biocatalyst owing to favourable properties such as thermostability and stability in common laccase inhibitors. However, the enzyme has a low redox potential and low activity, which tend to limit its substrate range. In the present study, we investigated the potential of inserting multiple/composite mutations by site-directed mutagenesis as a way of improving catalytic properties of the enzyme. Ten composite mutants were successfully constructed, and the recombinant mutant proteins were overexpressed in *E. coli* BL-21 (DE3) and purified by Ni^{2+}-NTA chromatography. Evaluation of kinetic parameters showed that two mutants, Y230A/V290N and Y229A/M198G had better catalytic properties than the wild type small laccase (WT-SLAC). Y230A/V290N showed the lowest K_m value of 0.122 mM (ABTS), which was approximately 75% lower than that of the WT-SLAC. The other mutant, Y229A/M198G showed a 1.2-fold enhancement in catalytic efficiency (k_{cat}/K_m) than WT-SLAC (ABTS). The two mutants also showed improved dye decolourisation capability compared with the WT-SLAC. The results demonstrate that combining single mutations can be a viable option for generating a novel biocatalyst with improved properties.

3.1 Introduction

Laccases are relevant biocatalysts due to their ability to oxidise a wide range of substrates (Shao *et al.*, 2009). The versatile oxidative ability of laccases has been exploited in myriad applications such as remediation of industrial effluents, wood delignification, organic syntheses, and the polymerisation of plant flavonoids for synthesis of bioactive compounds (Sherif *et al.,* 2013). Furthermore, unlike other oxidoreductases, laccases do not require expensive cofactors such as NADH and NADPH (Koschorreck *et al*., 2009). Laccases are also termed "green catalysts" since they only require molecular oxygen and produce water as the only by-product (Prins *et al.*, 2015) .

Fungal laccases have been extensively exploited in industrial processes, owing to its high redox potential (Gunne and Urlacher, 2012). However, unlike bacterial laccases such as the small laccase (SLAC), laccases of fungal origin are unstable at high temperatures and extreme pH levels (Fang *et al.*, 2014). Therefore, large scale application of fungal laccases is marred by the cost of enzyme production along with the instability of the enzyme under various industrial conditions (Kunamneni *et al.*, 2008)**.** In this respect, the SLAC can be a useful alternative, as it is has some desirable properties such as thermostability and activity over a wide pH range (Machczynski *et al.*, 2004). However, low enzymatic activity of SLAC could prove to be problematic in industrial applications (Chen *et al.*, 2017).

Among the existing alternative strategies in protein engineering for improving catalytic or biochemical properties is site-directed mutagenesis (SDM), a strategy that has been exploited in modifying several industrial enzymes. However, the important aspect of SDM is the selection of mutation sites (Yang *et al.*, 2013). Availability of the crystal structure of SLAC (Skálová *et al.,* 2009) has made it possible to mutate the key residues of this biocatalyst for

potential improvement of properties (Sherif *et al.*, 2013). Several important mutations such as V290N (Prins *et al.,* 2015), M168G, Y229A and Y230A (Sherif *et al.,* 2013) have been investigated by different research groups. These mutations reportedly improved catalytic efficiency of SLAC. Combining the mutations has potential to further enhance properties of the enzyme as observed in the *Bacillus pumilus* CotA-laccase (Chen *et al.*, 2017) and in *Phaseolus vulgaris* epoxide hydrolase (Li *et al.,* 2019).

In the present investigation, we attempted to combine single mutations that have improved properties of SLAC (Prins *et al.,* 2015; Sherif *et al.,* 2013; Toscano *et al.,* 2013) to construct composite mutants including double, triple and quadruple mutants via SDM to further improve the properties of the SLAC from *S. coelicolor* A3(2). The generated mutants were evaluated for improved substrate affinity, turnover number and catalytic efficiency. In addition, the ability of the mutants to decolourise common industrial dyes was compared to the wild-type SLAC (WT-SLAC).

3.2 Materials and methods

3.2.1 Materials

All chemical reagents were of analytical grade. The growth media and reagents were purchased from Merck (South Africa) and Sigma-Aldrich (South Africa). The plasmid extraction kit was obtained from ThermoFischer Scientific (South Africa). ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and all other chemicals were purchased from Sigma-Aldrich (Durban, South Africa).

3.2.2 Bacterial strains, plasmid, and growth conditions

SLAC gene was amplified and was sub-cloned into pET20b + (Novagen, USA), an expression plasmid. *Escherichia coli* DH5α and BL-21 (DE3; Stratagene) were used as hosts for the cloning and protein expression, respectively. All strains were grown in Luria–Bertani (LB) medium at 37°C. A pET-20b (+) vector (Novagen) containing the gene coding for SLAC (pSLAC) (WP_003972284) cloned into *E. coli* BL-21(DE3) cells was kindly provided by Prof. Gerard Canters, Leiden Institute of Chemistry, University of Leiden, The Netherlands. *Escherichia coli* DH5α used for the construction and routine propagation of vectors was purchased from Invitrogen, USA.

3.2.3 Site-directed mutagenesis

pET20b (+) with the unmutated SLAC gene was used as a template for all ten mutations. Four sets of synthetic primers were designed to introduce double, triple or quadruple mutations. Sense and antisense primers are as follows:

Y229A, F 5'- TCATGATCACGCACGGGGAG**GCG**TACCAC 3'

Y229A, R 5'- GTGGTACGCCTCCCCGTGCGTGATCATGA3'

Y230A, F 5- CACGCACGGGGAGTAC**GCG**CACACCTTCCA 3'

Y230A, R 5- TGGAAGGTGTGCGCGTACTCCCCGTGCGTG 3

V290N, F 5- ATGTACCACTGCCAC**AAC**CAGAGCCACT 3'

V290N, R 5- AGTGGCTCTGGTTGTGGCAGTGGTACAT 3'

M198G, F 5'- TCGTCTTCAACGAC**GGC**ACCATCAACAAC3'

M198G, R 5'- GTTGTTGATGGTGCCGTCGTTGAAGACGA 3'

The double-mutants **(**Y229A/V290N, Y230A/V290N, Y229A/Y230A, Y229A/M198G, Y230A/M198G and V290N/M198G), triple-mutants (Y229A/Y230A/V290N, Y229A/V290N/M198G and Y230A/V290N/M198G) and a quadruple-mutant (Y229A/Y230A/V290N/M198G) were constructed by PCR using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The mutated plasmid was transformed into competent *E. coli* BL-21 (DE3) cells. Furthermore, positive clones were selected by colony PCR and sent for sequencing (Inqaba, South Africa) to ensure that the correct mutation had been introduced.

3.2.4 Expression and purification of small laccase and its mutants

Escherichia coli BL-21 (DE3) cells harbouring the WT-SLAC and the mutated SLAC mutants were grown in 10 ml LB medium supplemented with ampicillin (100 µg/ml) at 37°C with shaking (200 rpm). Subsequently, the overnight grown culture was inoculated into a fresh LB medium (1% inoculation) with ampicillin and incubated at 37 °C with shaking (200 rpm) until the OD_{600} reached 0.5-0.6. Thereafter, protein expression was induced by 0.8 mM IPTG and further incubated at room temperature (25°C). Cells were harvested after overnight growth by centrifugation (10000 x g, 15 min, room temperature) and resuspended in Bugbuster Protein Extraction Reagent (Merck, South Africa). Thereafter, buffer was added and the lysates were centrifuged (10000 x g, 30 min; 4°C) and the supernatant was collected for further analysis.

Analysis of enzyme expression from the WT-SLAC and its mutants was performed using the ABTS solid plate assay. ABTS (2 mM) was added to sterile agar (2%) medium prepared in sodium acetate buffer (50 mM, pH 4.0). Cell free supernatant was added to wells made using a sterile stock borer and the plates were incubated at 50 °C for 30 min. The appearance of green halos around the wells was interpreted as positive laccase activity.

Expressed WT-SLAC and SLAC mutants have a 6× His tag at the C-terminus which enabled the purification by affinity column chromatography. The supernatant was loaded onto the nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin (Invitrogen, Life Technologies, USA) which was pre-equilibrated with binding buffer (100 mM Tris-Cl + 150 mM NaCl pH 8.0). Resin was washed twice using 8 ml of washing buffer (100 mM Tris-Cl + 100 mM NaCl + 20 mM imidazole). Thereafter, elution was performed using 8 ml elution buffer (100 mM Tris-Cl, 300 mM NaCl, 250 mM imidazole). Aliquots showing laccase activity (activity was measured using ABTS plate assay), were pooled together and desalted using Amicon ultrafiltration (membrane cut-off 10 kDa, Millipore, USA). In order to reuse and recharge the Ni-NTA column, washing was performed using EDTA (0.5M, pH 8.0) to strip off Ni^{2+} ions from the matrix, followed by MilliQ washing (10 column volumes). Thereafter, resin was loaded with three column volumes of nickel hexahydrate ($NiCl_2.6H_20$; 0.1M, pH 8.0) followed by MilliQ washing to remove excess Ni^{2+} ions. Thereafter, the resin was equilibrated with binding buffer and the protein was loaded onto the Ni-NTA matrix for a subsequent purification.

Protein concentration was estimated using Lowry assay (Lowry *et al*., 1951) using bovine serum albumin as the standard. The purity of the purified protein was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by staining with Coomassie Brilliant Blue R-250. The apparent molecular weight of the recombinant proteins was estimated by comparing with a standard marker which was loaded along with the proteins.

3.2.5 Laccase activity and protein determination

Laccase activity is measured using ABTS as a substrate as previously described by Chen *et al.* (2017) with slight modifications. Oxidation of ABTS (1 mM) was determined at 420 nm (ε =

36,000 M^{-1} cm^{-1}). Enzyme activity was defined as the amount of enzyme that oxidised 1 μmol of substrate per minute at the assay conditions. The Lowry method was used to determine the protein content with bovine serum albumin as a standard (Lowry *et al.*, 1951).

3.2.6 Biochemical characterisation

3.2.6.1 Optimum pH and temperature

The optimum pH for ABTS was determined by performing enzyme assays in different buffers with pH value ranging from 2.0-9.0 at 60 °C. The series of buffers used were as follows: [20 mM glycine-HCl buffer (pH 2.0, 3.0), 20 mM sodium acetate buffer (pH 4.0, 5.0), 20 mM Tris–HCl buffer (pH 6.0–8.0) and 20 mM glycine-NaOH buffer (pH 9.0)]. The optimum temperature was determined by conducting the enzyme assay at different temperatures (50°C–90°C) at the optimum pH for ABTS.

3.2.6.2 Enzyme kinetics

Kinetic parameters (K_m, v_{max}, k_{cat} and k_{cat}/K_m) for WT-SLAC and its mutants were determined at 80 °C and pH 4 using ABTS as substrate in the concentration range of 10−1000 μM. The data was fitted to the Lineweaver Burk plot to determine the kinetic parameters. All the assays were performed in triplicate.

3.2.7 Dye decolourisation

Crude supernatant was used to assess the decolourisation potential of the improved mutants as well as WT-SLAC against three groups of dyes: triphenylmethane dyes namely Brilliant Blue G (BBG; λmax = 630 nm), Acid Red 94 (AR94; λmax = 546 nm), Malachite Green (MG; λmax = 617 nm), and Brilliant Green AR (BGAR; λmax = 625 nm); azo dyes namely Trypan Blue (TB; λmax = 600 nm), Reactive orange 16 (RO16; λmax = 495 nm), and Methyl Red (MR;

λmax = 540 nm); and an anthraquinone dye namely Remazol Brilliant Blue R (RBBR, λmax = 590 nm).

The reaction mixture (800 µL) contained 0.078 U/mL crude laccase, 50 mg/L dye when MR, MG or BGAR were used, or 75 mg/L of RO16 or 150 mg/L of RBBR, 0.05 M of $CuSO_4$, redox mediator (acetosyringone /ABTS/ HBT; 2 mM) and 50 mM sodium acetate buffer (pH 4.0) and was incubated overnight shaking (150 rpm) at 37 °C. The decolourisation potential was assessed spectrophotometrically as the relative decrease in the absorbance at the maximum absorbance wavelength of the dyes. All the reactions were carried out in triplicate. A control reaction was also performed in parallel where a crude supernatant from vector control was used.

3.3 Results and Discussion

3.3.1 Design, cloning and expression of SLAC mutants

This study was aimed at enhancing the catalytic properties of the SLAC (from *S. coelicolor* A3(2)) through site-directed mutagenesis. Mutant site selection is the crucial part of site-directed mutagenesis (Xu *et al.*, 2019), as a single amino acid can have a critical or negligible effect on enzyme properties depending on its position in a protein structure (Akbarzadeh *et al.*, 2018). Several previous studies have been conducted on SLAC from *S. coelicolor* A3(2) to improve its turnover number (Prins *et al.*, 2015), and relative activity (Sherif *et al.*, 2013; Toscano *et al.*, 2013). These studies led to the identification of single mutations (Y229A, Y230A, V290N and M198G) which had shown favourable catalytic properties. However, there has been no study that made an effort to study the additive effect of combining these single mutations. Therefore, in the present study an attempt was made to combine these single mutations to enhance the properties of the SLAC. A total of ten composite mutants were

generated including six double mutations (Y229A/Y230A, Y229A/V290N, Y230A/V290N, V290N/M198G, Y230A/M198G and Y229A/M168G), three triple mutations (Y229A/Y230A/V290N, Y229A/V290N/M198G and Y230A/V290N/M198G) and one quadruple mutation (Y229A/Y230A/V290N/M198G), via site-directed mutagenesis. The expression from BL-21 (DE3) cells harbouring these mutants were tested qualitatively using ABTS plate assay **(Fig. 3.1)** The mutants displaying positive laccase activity (green halo around the wells) were then purified by Ni^{2+}-NTA affinity column.

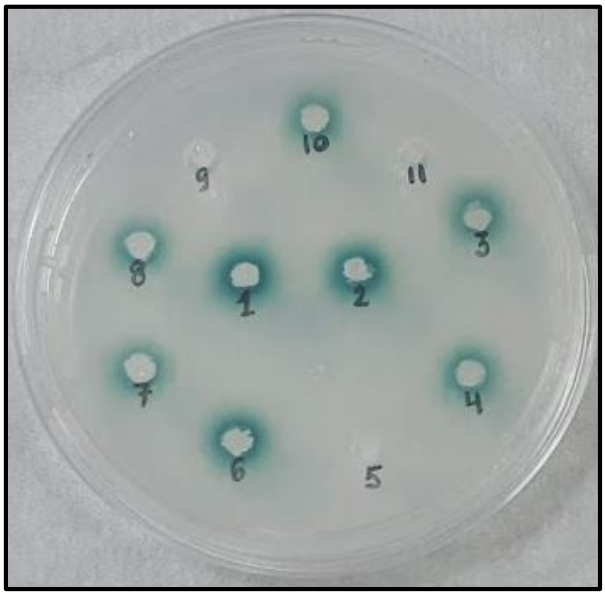


Fig. 3.1 ABTS plate assay performed from the protein obtained from *E. coli* harbouring wild type SLAC (1) and SLAC mutants: Y229A/Y230A (2), Y229A/V290N (3), Y230A/V290N (4), V290N/M198G (5), Y230A/M198G (6), Y229A/M198G (7), Y229A/Y230A/V290N (8), Y229A/V290N/M198G (9), Y230A/V290N/M198G (10) and Y229A/Y230A/V290N/M198G (11).

3.3.2 Purification and characterisation of the mutants

Nickel ion affinity chromatography enabled one-step purification of WT-SLAC and its mutants to homogeneity as depicted by SDS-PAGE analysis **(Fig. 3.2)**. A predicted molecular weight

of ~38 kDa was observed for WT-SLAC and all the mutants (**Fig. 3.2**). This was due to the fact that the proteins (37 kDa) were fused with the His-tag (6 his units or 1 kDa).

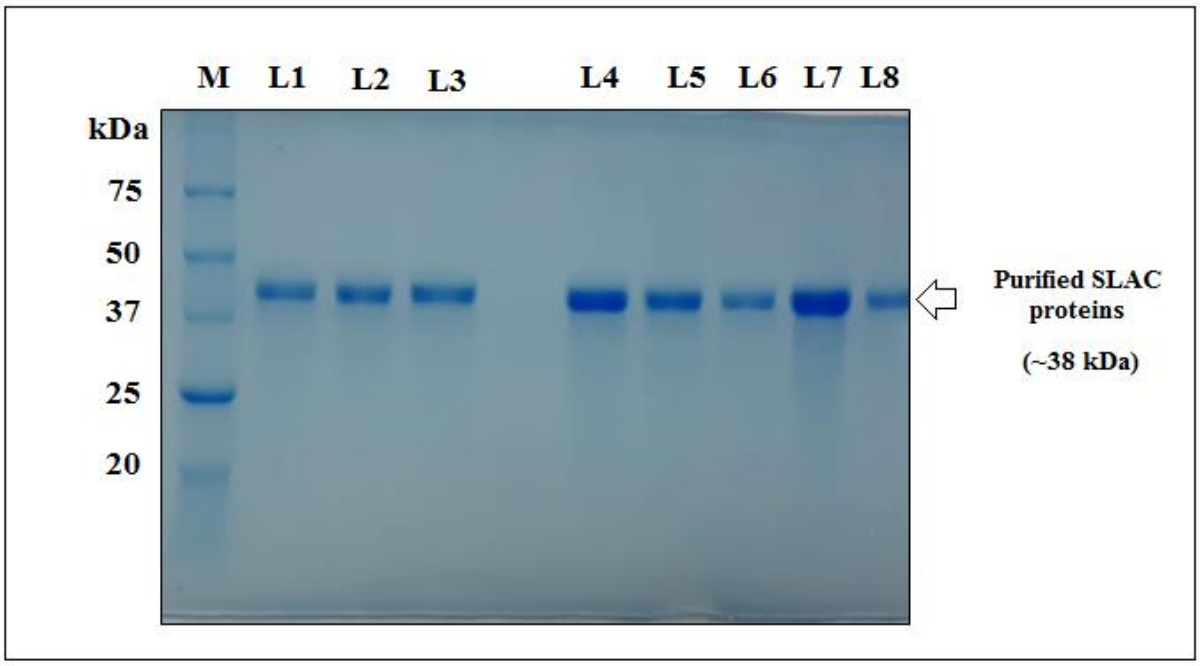


Fig. 3.2 SDS-PAGE analysis of purified WT-SLAC and its mutants. Lane M: protein marker; L1 to L8: WT-SLAC (L1) and its mutants; Y229A/V230A (L2), Y229A/V290N (L3), Y230A/V290N (L4), Y230A/M198G (L5), Y229A/M198G (L6), Y229A/Y230A/V290N (L7) and Y230A/V290N/M198G (L8).

Assessment of pH showed an optimum of 4.0 with ABTS for purified WT-SLAC and all its mutants (**Fig. 3.1a**). All enzymes showed a rapid decrease of activity at pH 3 and 5. Meanwhile, WT-SLAC and the mutants revealed the same optimum temperature at 80 °C with ABTS as substrate (**Fig. 3.1b)**. However, some mutants showed lower activity at 90 °C when compared to the WT-SLAC. This implies that they could be less stable at higher temperature than WT-SLAC.

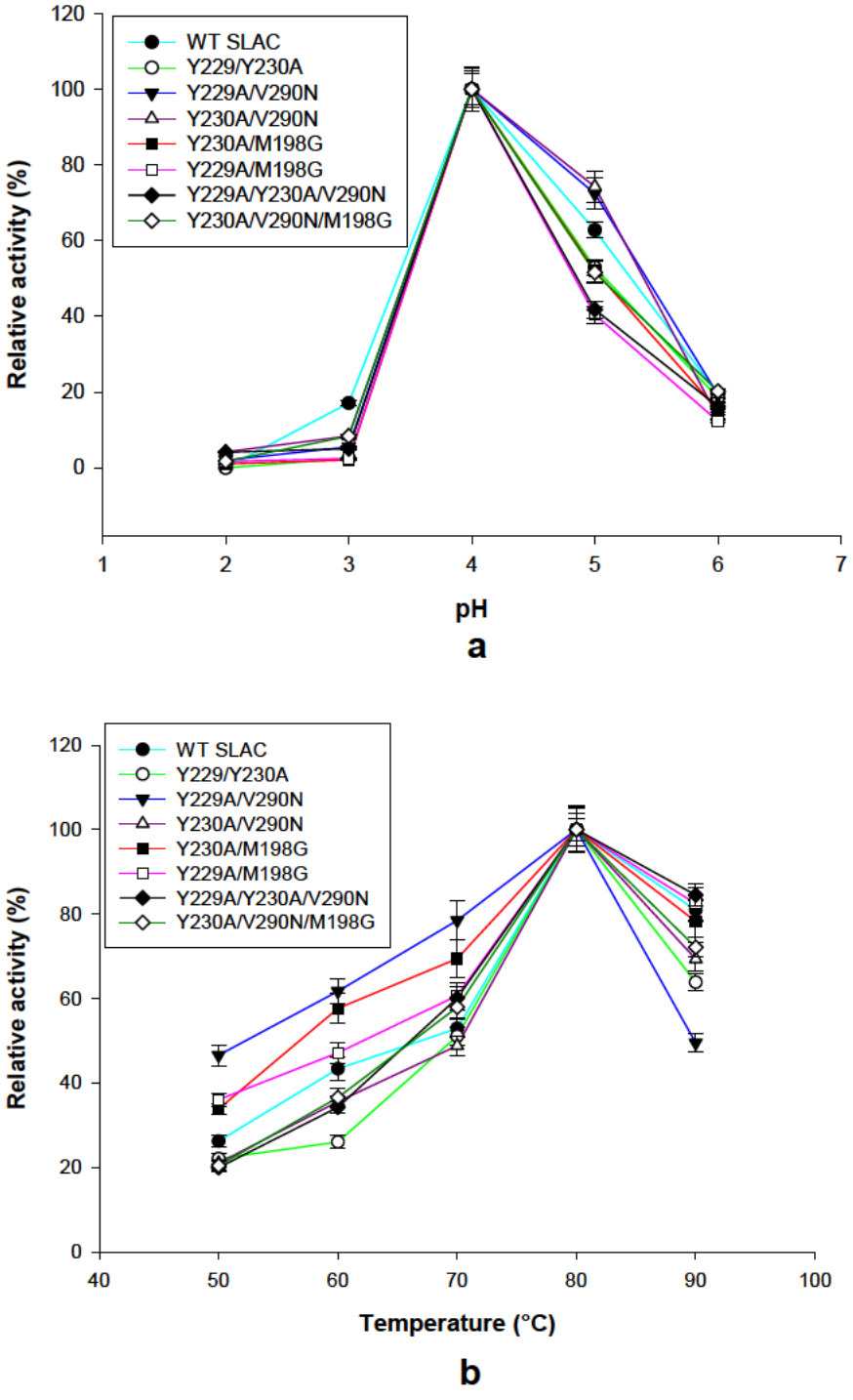


Fig. 3.3 Optimum (a) pH and (b) temperature of WT-SLAC and its mutants using ABTS as a substrate.

ABTS, a typical laccase substrate was used for characterising the catalytic properties of WT-SLAC and its mutants. The kinetic parameters specifically substrate affinity (K_m), turnover rates (k_{cat}) and catalytic efficiency (k_{cat}/K_m) are given in **Table 3.1**.

Substrate orientation (Monza *et al.*, 2015) at the active site as well as electron transfer to the T1 Cu site (Jones and Solomon, 2016) have been known to have an effect towards enhanced activity and turnover rate respectively. Therefore, in the present investigation, we shortlisted four mutations related with substrate binding site (Y229A, Y230A) and T1 Cu site (V290N, M198G) and combined those mutation to determine whether combining single mutants had any additive effect. Analysis of kinetic parameters revealed that all the mutants showed lower K_m values than the WT-SLAC. The lowest K_m was observed for Y230A/V290N which was 75.50% lower than the WT-SLAC. A lower K_m value is indicative of higher affinity towards the investigated substrate (i.e. ABTS). In addition, turnover rate (k_{cat}) was also calculated and it was found that k_{cat} value was lower than WT-SLAC for all the mutants. Additionally, catalytic efficiency (k_{cat}/K_m) or specificity constant of double mutations were found to be closer or slightly lower than WT-SLAC except for Y229A/M198G (1.2-fold higher than that of the WT-SLAC). However, triple mutations showed much lower catalytic efficiencies. This seems consistent with another report (Kataoka *et al.*, 2013) where a triple mutation lead to a considerable decrease in turnover number. However, in another study performed by Chen *et al.* (2017), a composite triple mutant exhibited enhanced thermostability and catalytic efficiency, but the experiments on dye decolourisation didn't show any significant difference when compared with the evolved double mutant.

A comparison with the existing data in the literature for single mutants indicate that combining the mutations resulted in a decrease in turnover rate (k_{cat}) and catalytic efficiency (k_{cat}/K_m),

however, substrate affinity (K_m) improved considerably (Sherif *et al.*, 2013; Toscano *et al.*, 2013; Prins *et al.*, 2015) For example, K_m values were better than those of the single site mutation V290N (0.285 mM) (Prins *et al.*, 2015). It is worth noting that the single mutants V290N and Y229A have been associated with an alteration of substrate binding and increased substrate specificity (Sherif *et al.*, 2013; Prins *et al.*, 2015). On the other hand, M168G (M198G in this study because the SLAC sequence used in the present study had the signal peptide; the first 30 amino acids form the signal peptide in the SLAC gene sequence) has been associated with an increase in catalytic efficiency (over the wild-type enzyme) as well as improved K_m (10-fold lower) (Toscano *et al.*, 2013). Removing the Met residue resulted in an increased accessibility to the active site and reduction of the distance between substrate and the T1 Cu site thereby enhancing the transfer of electrons and the reaction rate (Toscano *et al.*, 2013). The values obtained for catalytic efficiency (k_{cat}/K_m) using Y229A/M198G in this study were also found to be superior than those obtained for CotA from *Bacillus subtilis* (Khodakarami *et al.*, 2018).

Table 3.1 Kinetic constants for WT-SLAC and its mutants (ABTS used as substrate)

Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)
WT-SLAC	0.498	3.72	7.46
Y229/Y230A	0.220	1.57	7.13
Y229A/V290N	0.180	1.03	5.72
Y230A/V290N	**0.122**	0.77	6.31
Y230A/M198G	0.210	1.52	7.20
Y229A/M198G	0.378	3.45	**9.12**
Y229A/Y230A/V290N	0.232	1.08	4.60
Y230A/V290N/M198G	0.239	0.79	3.30

All assays were performed at optimal pH for each enzyme (buffer ionic strength: 50 mM), at 80 °C.

3.3.3 Dye decolourisation

Dyes from three different classes (triphenylmethane, azo and anthraquinone) were selected as the diverse structures of the dyes would enable us to assess the substrate specificity of the mutants. The mutant exhibiting the lowest K_m value (i.e. Y230A/V290N) was selected for its potential application in dye decolourisation of eight industrial dyes as well as to determine whether the substrate specificity improved. **Fig. 3.4a** shows that both WT-SLAC and Y230A/V290N were able to completely decolourise BBG, AR 94 and TB without the aid of any mediators. However, redox mediators were included during the decolourisation of the other dyes. Inclusion of redox mediators enabled the decolourisation of otherwise recalcitrant dyes. Optimisation for best redox mediators among ABTS, 1-hydroxybenzotriazole (HBT), acetosyringone (AS) and syringaldehyde (SYR) for each dye was investigated. ABTS was

found to be the best redox mediator for MG and BGAR. On the other hand, HBT and AS were the best mediators for the decolourisation of RO16 and MR, respectively **(data not shown)**.

The mutant Y230A/V290N showed superior potential in decolourising the selected dyes (viz. MR, MG, BGAR, RBBR and RO16) when compared with WT-SLAC. It is worth noting that the single site mutations such as Y229A and Y230A are on the substrate binding surface and they have been associated with altered substrate binding as well as greater incorporation of copper than their wild type counterparts. Higher incorporation of copper tends to increase the activity of the enzyme (Sherif *et al.*, 2013). Furthermore, mutation V290N was reported to cause increased substrate affinity along with a decrease in the turnover rate using a non-phenolic substrate (Prins *et al.*, 2015). We also observed improved decolourisation potential using the mutant (Y229A/M198G) exhibiting superior catalytic efficiency **(Fig. 3.4b)**. Complete decolourisation of 50 mg/L of MR was achieved within 5 h with 0.078U/ml of the enzyme (Y229A/M198G). However, with Y230A/V290N, around 58.12% of MR was decolourised in 5 h. The WT-SLAC could only decolourise 28.35% of MR in 5 h. The better results obtained with the mutant, Y229A/M198G, could be attributed to the high catalytic efficiency of the mutant (1.2-fold higher than WT-SLAC). Increased decolourisation potential of mutants can also result from changed electrostatic interaction between the catalytic residues and redox mediator or favourable hydrophobic interactions (Chen *et al.*, 2017).

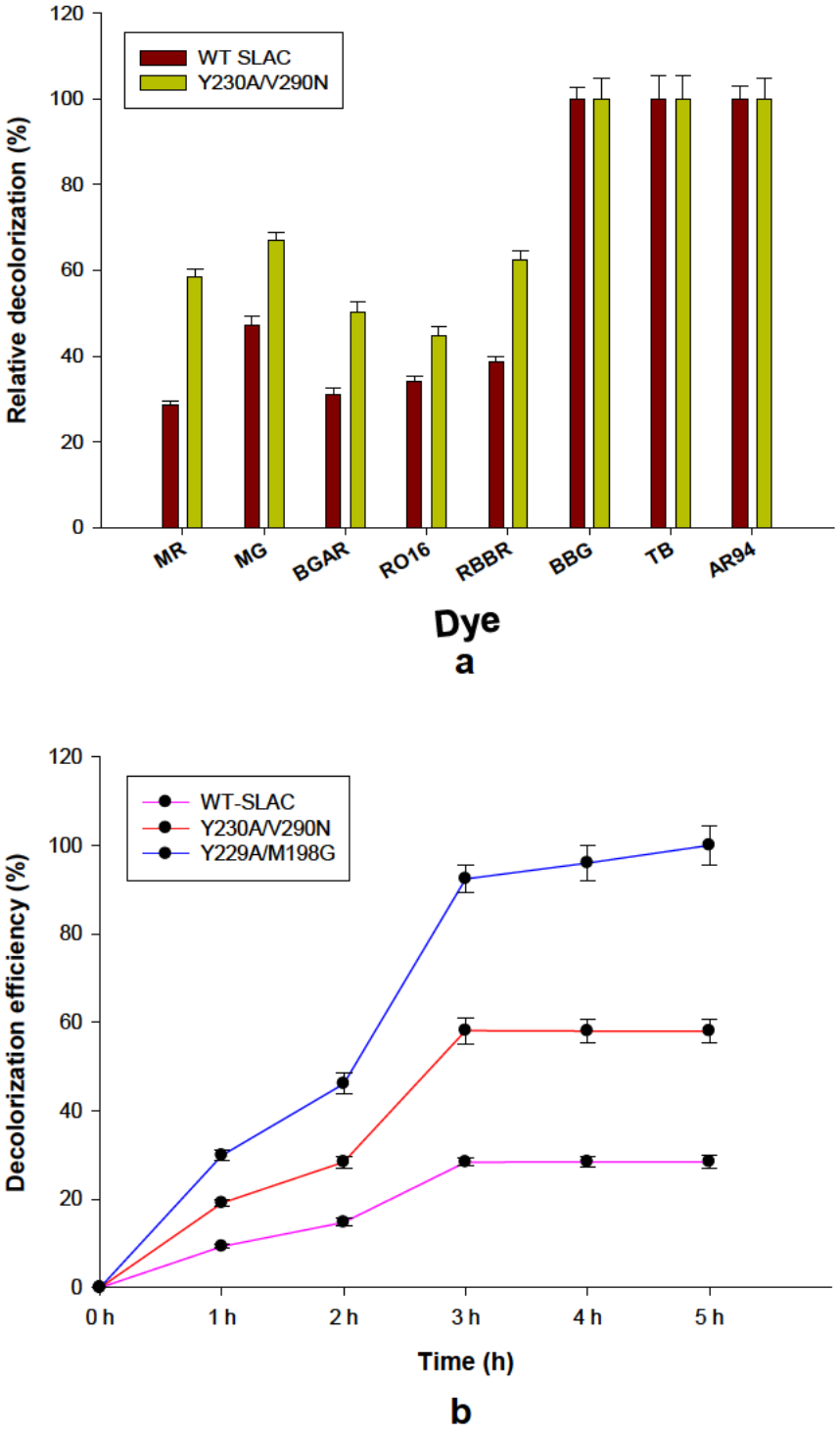


Fig. 3.4 (a) Decolourisation of synthetic dyes using crude WT-SLAC and mutant Y230A/V290N at pH 4.0 for 8 h at 37 °C. The bars represent the mean ± standard deviation of triplicate determinations; **(b)** Decolourisation of methyl red by WT-SLAC, Y230A/V290N and Y229A/M198G.

3.4 Conclusions

In summary, two double mutants (Y230A/V290N and Y229A/M198G) exhibited improved kinetic parameters, as well as improved ability to decolourise different classes of industrial dyes in the presence of mediators (AS, ABTS and HBT) compared to the wild-type enzyme. The results demonstrated that combining the single site mutations could overcome some of the limitations of SLAC by creating improved biocatalysts with practical industrial applications.

CHAPTER 4

Secretory expression of recombinant small laccase from *Streptomyces coelicolor* A3(2) in *Pichia pastoris*

(*Published*: Yadav, D., Ranjan, B., Mchunu, N., Le Roes-Hill, M. and Kudanga, T., 2018. Secretory expression of recombinant small laccase from *Streptomyces coelicolor* A3 (2) in *Pichia pastoris*. *International Journal of Biological Macromolecules*, *108*, pp.642-649)

Abstract

This work reports for the first time the secretory expression of the small laccase (SLAC) from *Streptomyces coelicolor* A3(2) in *Pichia pastoris.* Using an AOX1 promoter and α factor as a secretion signal, the recombinant *P. pastoris* harbouring the laccase gene (*rSLAC*) produced high titres of extracellular laccase (500 ± 10 U/l), which were further increased seven-fold by pre-incubation at 80 ºC for 30 min. The enzyme (~38 kDa) had an optimum activity at 80 ºC, but optimum pH varied with substrate used. K_m values for ABTS, SGZ and 2,6-DMP were 142.85 μM, 10 μM and 54.55 μM and the corresponding k_{cat} values were 60.6 s^{-1}, 25.36 s^{-1} and 27.84 s^{-1}, respectively. The $t_{1/2}$ values of the rSLAC at 60 ºC, 70 ºC, 80 ºC were 60 h, 32 h and 10 h, respectively. The enzyme deactivation energy (E_d) was 117.275 kJ/mol while ΔG, ΔH and ΔS for thermal inactivation of the rSLAC were all positive. The rSLAC decolourised more than 90% of Brilliant Blue G and Trypan Blue dye in 6 h without the addition of a mediator. High titres of SLAC expressed in *P. pastoris* enhances its potential for various industrial applications.

4.1 Introduction

Laccase is a multicopper oxidase, capable of catalysing one-electron oxidation of a wide range of substrates such as diphenols, methoxy-substituted monophenols, aromatic and aliphatic amines to generate radicals while concomitantly reducing molecular oxygen to water (Kudanga and Le Roes-Hill, 2014). Laccases are widely distributed in nature; they have been isolated from fungi, plants, insects and bacteria (Mate and Alcalde, 2015) of which fungal laccases are well characterised (Baldrian, 2005). Currently most applications are carried out with fungal laccases. However, applicability of fungal laccases is hindered in industrial processes which operate at harsh conditions of temperature, pressure or extremely alkaline pH (Hilde, 2009; Turner *et al*., 2007). Moreover, it is worth noting that fungal laccases show optimum activity at an acidic pH range and laccase catalysed oxidation efficiency is lowered with increase in pH due to OH^- ligation to the tri-nuclear T2/T3 cluster which prevents the internal transfer of electron from T1 copper to the T2/T3 cluster (Zucca *et al.*, 2016).

Bacterial laccases, such as the small laccase from *S. coelicolor* (SLAC), can be useful alternatives to fungal laccases. SLAC exhibits some desirable characteristics such as activity at high temperatures and alkaline pH (Dubé *et al*., 2008), making it a potential candidate in biotechnological applications. However, low production yield by native microbial hosts (Ranjan and Satyanarayana, 2016) is a major bottleneck for commercial applications (Fan *et al*., 2015). Consequently, a vast amount of research has focused on the search for simple heterologous expression systems, with *Escherichia coli* as the most frequently used expression system (Santhanam *et al.*, 2011). Features such as availability of established bacterial genetic tools and biotechnological processes, plasmid DNA stability and high transformation efficiency make *E. coli* the most sought after host system (Santhanam *et al.*, 2011; Sharma *et al*., 2007). However, overexpression in *E. coli* is often limited by intracellular aggregation

(Fang *et al.*, 2014; Rosano and Ceccarelli, 2014) leading to significantly low yields of soluble protein (Chen *et al.*, 2015; Fang *et al*., 2011; Martins *et al*., 2002; Suzuki *et al.*, 2003). In addition, inclusion bodies are also difficult to purify thereby increasing production costs (Santhanam *et al*., 2011).

Extracellular expression could be a desirable alternative for expression of foreign proteins since it simplifies purification steps. *P. pastoris* is usually the preferred host for the production of industrial enzymes (Ahmad *et al*., 2014). It offers several advantages over other recombinant expression hosts such as ability to grow at high cell densities, low levels of native proteins, no antibiotic requirement once a stable integrant is obtained, and availability of expression vectors which utilise methanol as an inexpensive carbon source and inducer (Joshi and Satyanarayana, 2015; Yang *et al*., 2015). *P. pastoris* has been successfully used in expressing high yields of recombinant proteins through high cell density cultivation (Mchunu *et al*., 2009). Hence, using an expression plasmid with α-factor signal sequence can prove to be useful in enhancing the production of SLAC.

In this chapter, the research was focused on heterologous expression of SLAC gene from *S. coelicolor* in *Pichia pastoris*. The recombinant protein (rSLAC) was purified, characterised for its kinetic and thermodynamic parameters. In addition, the decolourisation ability of the rSLAC on Brilliant Blue G and Trypan Blue, was investigated.

4.2 Materials and Methods

4.2.1 Materials

2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), syringaldazine (SGZ), Brilliant Blue G, and Trypan Blue were purchased from Sigma-Aldrich (South Africa).

4.2.2 Microorganisms and media

A pET-20b (+) vector (Novagen) containing the gene coding for SLAC (pSLAC) (WP_003972284) was cloned into *E. coli* BL21(DE) cells and was kindly provided by Prof. Gerard Canters, Leiden Institute of Chemistry, University of Leiden, The Netherlands. *E. coli* DH5α used for the construction and routine propagation of vectors was purchased from Invitrogen. *P. pastoris* GS115 used for the expression of recombinant vector was obtained from Invitrogen. Yeast extract peptone dextrose (YPD), yeast extract peptone (YP), buffered glycerol-complex medium (BMGY), and buffered minimal methanol medium (BMM) were used to culture the methylotrophic yeast, *P. pastoris* and were prepared according to the manual of Easy select *Pichia* expression kit (Invitrogen).

4.2.3 Cloning of *SLAC* gene from *Streptomyces coelicolor*

SLAC encoding gene was amplified using the forward primer containing the *Eco*RI site and reverse primer containing the *Xba*I site (**Table 4.1**). PCR reaction was conducted with the following cycling conditions: Preheating 95 ºC for 1 min, followed by 35 cycles of (95 ºC for 40 s, 55 ºC for 30 s, 72 ºC for 2 min), and a final extension at 72 ºC for 10 min. The resulting PCR product was double digested by *EcoR*I and *Xba*I and purified by GeneJet purification Kit (Thermofischer scientific). The digested PCR product was then ligated into digested pPICZαA

vector and transformed into *E. coli* DH5α. Transformants were selected on low salt LB supplemented with 25µg/ml zeocin.

Table 4.1 Primers used in this study; underlines indicate restriction site added to the primers

Primers	Oligonucleotide Sequence (5′ → 3′)	Restriction site
SLAC F′	CGGAATTCATGGACAGGCGAGGCTTTAA	*Eco*RI
SLAC R′	CGTCTAGATAA GTGCTCGTGTTCGTGTGCGG	*Xba*I

GAATTC, restriction site for *Eco*RI; GGTACC, TCTAGA, restriction site for *Xba*I

4.2.4 Transformation of *Pichia pastoris* and screening of recombinant clones

Positive recombinant (*pPICZαA-SLAC*) was linearised using *Sac*I for transformation in *P. pastoris* GS115 through electroporation. Approximately 10 µg of linearized plasmid was added to yeast competent cells and pulsed at 1.5 kV, capacitance of 25 µF and 200 Ω of resistance. The cells were recovered with 1.0 M sorbitol and the mixture was transferred on YPD agar (g/l: yeast extract 10, peptone 20 and dextrose 20, agar 20) medium supplemented with 1.0 M sorbitol containing different concentrations of zeocin (100, 200, 500, 1000 and 1500 µg/ml). The plates were incubated at 30 °C until single distinct colonies appeared. Colony PCR was performed to identify positive transformants (Ranjan and Satyanarayana, 2016). The positive clones were again transferred to fresh YPD agar plates (supplemented with 1.0 M sorbitol) with varying concentrations of zeocin (as described above). Small scale expression trials were performed to screen for the best producer of rSLAC. The transformants were first grown in YPD broth and then the biomass was pelleted (1500 x g, 10 min) and transferred to YP medium consisting of 0.5% (v/v) methanol to an OD_{600} of 1.0. The culture was cultivated at 30 ºC, 250 rpm with 0.5% (v/v) methanol being added every 24 hours for 7 days. The culture supernatant was harvested by centrifugation at 4000 × *g* for 10 min at 4 ºC and quantitative laccase assay was performed. The best clone was selected for further optimisation.

4.2.5 Optimisation of rSLAC production

Various parameters such as the different methanol concentration (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) and different media composition (YP and BMMY) were tested to determine the contribution of each parameter in overproduction of rSLAC. The cell biomass was generated in YPD/ BMGY for YP/BMMY, respectively. Recombinant *P. pastoris* was inoculated in 50 ml YPD/BMGY medium in 250 ml conical flasks and was cultivated at 30 ºC, 250 rpm until the OD_{600} reached 2.0-6.0. The cells were harvested during the log phase and added into the induction medium (YP/BMMY) to an OD_{600} of 1.0. The culture was incubated at 30 ºC, 250 rpm with continuous addition of methanol at 24-hour intervals to achieve concentrations ranging from 0.5 % - 3.0%. Samples (100 µl) were withdrawn every day and cell biomass and laccase activity was determined. The effect of two non-repressing carbon sources (mannitol and sorbitol) in culture medium in combination with regular methanol induction was also studied. All experiments were performed in triplicate and results expressed as mean ± standard deviation.

4.2.6 Purification of recombinant SLAC

Cell free culture medium (YP) was collected by centrifugation (4000 × *g* for 30 min). The crude enzyme (supernatant) was concentrated by ammonium sulphate precipitation (30-80%) at 4 ºC. The fractions were desalted by overnight dialysis (dialysis tubing; MW cut-off 14,000) and dialysed samples were incubated with $CuSO_4$ to restore enzymatic activity. The concentrated enzyme was then purified by fast protein liquid chromatography (FPLC) system [ÄKTApurifier 100, GE Healthcare, Bio-Sciences, Uppsala, Sweden] using HiTrap Capto Q column (GE Healthcare, Bio-Sciences, Uppsala, Sweden) and eluting the bound protein with a linear gradient of 0–1 M NaCl in Tris-HCl buffer (20 mM, pH 8.0) at 1.5 ml/min. The fractions

with laccase activity were pooled and dialyzed against Tris-HCl buffer (20 mM, pH 8.0) and concentrated using a 10 kDa amicon ultra-15 centrifugal filter. The concentrated enzyme was loaded on a gel filtration chromatography column [Sephacryl S-300HR (26/60)] and eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl at a flow rate of 0.8 ml/min. The fractions having laccase activity were collected and their purity was determined using SDS-PAGE. The purified enzyme was used for enzyme characterisation.

4.2.7 Determination of molecular mass

Molecular mass of the active fraction was determined by SDS-PAGE by comparing the electrophoretic mobility of the enzyme to that of the markers of known molecular weight. SDS-PAGE was carried out by using 12% resolving gel and 5% stacking gel, which was further stained by Coomassie brilliant blue G-250.

4.2.8 Laccase activity assay and protein determination

Laccase activity was determined using ABTS, SGZ and 2,6-DMP as substrates, as described previously (An *et al.*, 2015; Prins *et al.*, 2015). Oxidation of ABTS (1 mM) was measured at 420 nm ($\varepsilon = 36{,}000 M^{-1} cm^{-1}$) in 20 mM acetate buffer (pH 4.0). The oxidation of SGZ (0.1 mM) was determined at 525 nm ($\varepsilon = 65{,}000 M^{-1} cm^{-1}$) in Tris-HCl buffer (pH 8.0). The oxidation of 2,6-DMP (2 mM) was determined at 466 nm ($\varepsilon = 14{,}700\ M^{-1} cm^{-1}$) in Tris-HCl buffer (pH 8.0). The Lowry method was used to determine the protein content with bovine serum albumin as a standard (Lowry *et al.*, 1951)

4.2.9 Biochemical characterisation of rSLAC

The optimum pH for the activity of rSLAC was determined by performing assays in different buffers [20 mM glycine-HCl buffer (pH 2.0, 3.0), 20 mM sodium acetate buffer (pH 4.0, 5.0),

20 mM Tris–HCl buffer (pH 6.0–8.0) and 20 mM glycine-NaOH buffer (pH 9.0)] at 80 ºC. Optimum temperature was determined by conducting enzymatic assays at different temperatures (30 ºC-90 ºC) at the optimum pH for the substrate.

4.2.10 Enzyme kinetics and thermal deactivation of the rSLAC

Kinetic parameters of purified laccase were determined at 70 ºC and 80 ºC using different concentrations of ABTS (10-1000 μM), SGZ (5-100 μM) and 2,6-DMP (50-2000 μM). Assays were performed at each substrate's optimal pH (pH 4.0 for ABTS and pH 8.0 for SGZ and 2,6-DMP) and the K_m and k_{cat} values were determined. Activation energy (E_a) was calculated according to (Ranjan *et al*., 2015). The energy of deactivation (E_d) of the enzyme was calculated from residual activity at different temperatures by incubating the enzyme solution in 20 mM sodium acetate buffer (pH 4.0) for ABTS and 20 mM Tris-HCl buffer (pH 8.0) for SGZ and 2,6-DMP at various temperatures (60–80 °C) in the absence of substrate. Aliquots were drawn at the desired intervals and laccase assays performed for calculating the residual activities. Inactivation rate constants (K_d) and energy of deactivation (E_d) were calculated as explained below:

Thermal denaturation is a reaction in which the rate of enzyme deactivation (dC/dt) follows first order kinetics in relation to the concentration of the active enzyme (C):

$$dC/dt = -k_dC \quad (1)$$

which can also be expressed as

$$\ln[C_t/C_0] = -k_dt \quad (2)$$

where C_o is the initial concentration of the enzyme and C_t is the concentration of the enzyme at time t. Since the residual enzyme activity (E) is directly proportional to the concentration of the active enzyme (C), thus,

$E_t/E_0 = C_t/C_0$

Now equation (2) can be written as:

$\ln[E_t/E_0] = -k_d t$

or

$2.303\log[E_t/E_0] = -k_d t$ (3)

K_d is the deactivation rate constant, which is calculated from the slope of the plots of log $[E_t/E_0]$ vs. t. The half-life ($t_{1/2}$) of the enzyme is defined as the time required for the enzyme to lose half its initial activity. It can be expressed as follows:

$t_{1/2} = 2.303\log 2/K_d$ (4)

Energy required for deactivation (E_d) has been calculated by Arrhenius plot using the Arrhenius equation:

$K_d = Ae^{(-E_d/RT)}$ (5)

So that

$\ln[K_d] = -E_d/RT + \ln A$ (6)

where E_d represents energy of deactivation, R is the universal gas constant (8.314 J/K/mol) and T the absolute temperature. Deactivation energy (E_d) involved in the deactivation process is calculated from the slope of a linear plot of $\ln[K_d]$ vs T^{-1}. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a raise in the temperature by 10 °C. Temperature quotient (Q_{10}) was calculated by rearranging the equation of Dixon and Webb.

$Q_{10} = \text{antilog}\ (E_a \times 10/RT^2)$ (7)

Where E_a = activation energy, R is universal gas constant and T is absolute temperature.

4.2.11 Thermodynamic parameters of the rSLAC

Thermodynamics of irreversible inactivation of the rSLAC was determined by rearranging the Eyring's absolute rate equation derived from the transition state theory:

$K_d = (K_bT/h)\, e^{(-\Delta H/RT)}\, e^{(-\Delta S/RT)}$

ΔH (change in enthalpy of deactivation), ΔG (change in free energy of inactivation), and ΔS (change in entropy of inactivation) for irreversible inactivation were calculated as follows:

$\Delta H = E_d - RT$ (8)

$\Delta G = -RT \ln (K_dh/K_bT)$ (9)

$\Delta S = (\Delta H - \Delta G)/ T$ (10)

where K_b is the Boltzmann's constant (R/N) =1.38 x 10^{-23} J/K, T is the absolute temperature (K), h the Planck's constant = 6.626 x 10^{-34} J s, N is the Avogadro's number = 6.02 x 10^{23} mol^{-1}, R is the gas constant = 8.314 J/K/mol.

4.2.12 Decolourisation of dyes by rSLAC

The purified rSLAC was tested for the decolourisation of two synthetic dyes, Brilliant Blue G (**Fig. 4.1a**) (BBG, λ_{max} 630 nm) and Trypan Blue (**Fig. 4.2b**) (TB, λ_{max} 600 nm) in the absence of mediators. The reaction mixture (500 µl) consisted of 20 mM buffer (sodium acetate pH 4.0, Tris–HCl pH 6.0 and 8.0) containing dyes (50 mg/l, final concentration) and purified rSLAC (1 U/ml, activity in reaction mixture). The reaction was carried out at 37 ºC with mild shaking (50 rpm) for 12 h. In order to rule out the event of abiotic decolourisation, a control reaction was also run in parallel where a reaction was set up with the denatured enzyme (boiled for 30 min) under identical conditions. The decolourisation of Brilliant Blue G and Trypan Blue dyes were spectrophotometrically determined as the relative decrease of the maximum absorbance wavelength of 630 nm and 600 nm, respectively. Decolourisation assays were performed in

triplicate and percentage decolourisation was calculated according to (Jadhav *et al.*, 2007) as shown below:

% Decolourisation = [(I-F)]/ I] * 100

Where, I = initial absorbance and F = Absorbance after decolourisation

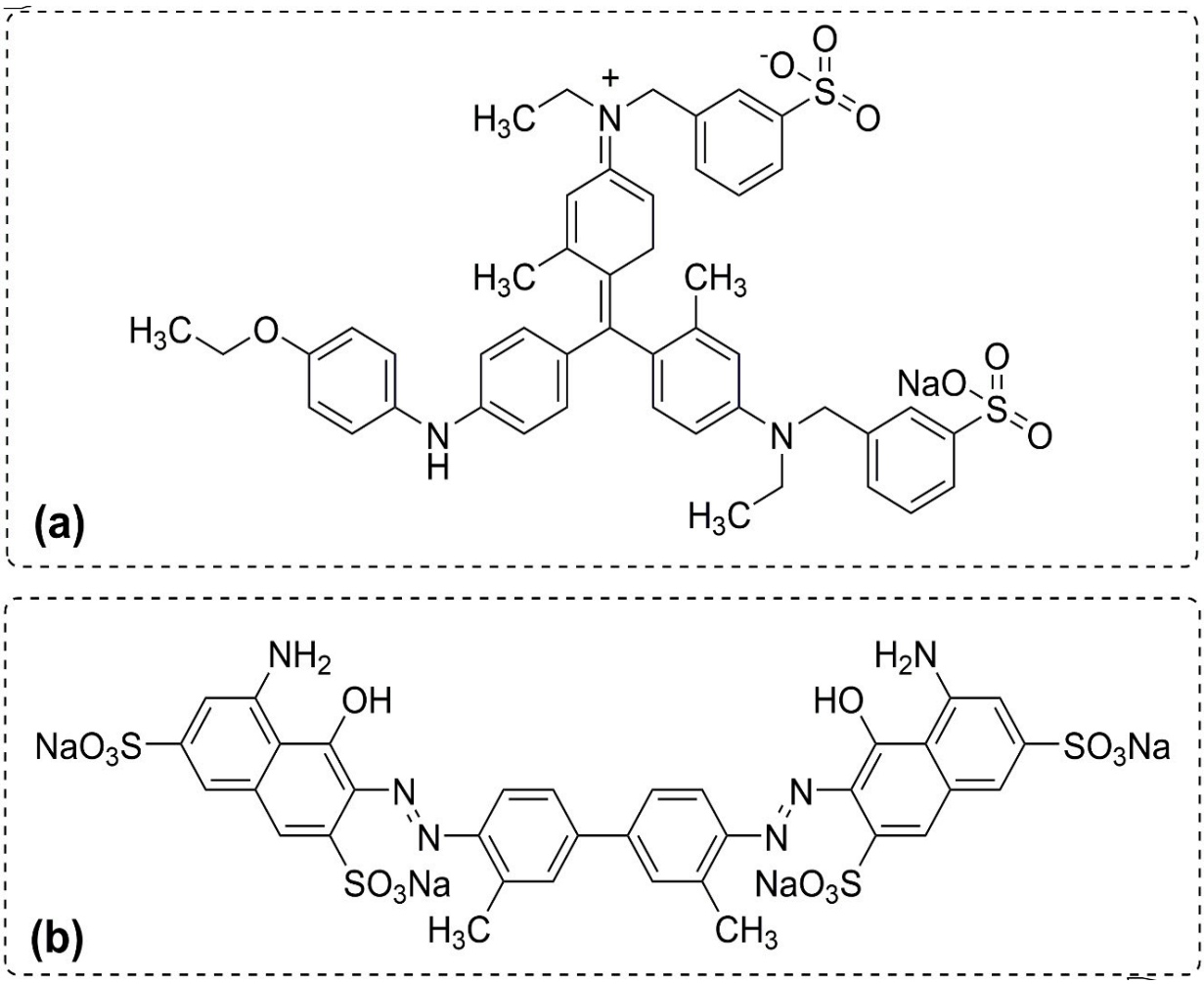


Fig. 4.1 Structure of dyes (a) Brilliant blue R; (b) Trypan blue (source: ChemDraw).

4.3. Results and discussion

4.3.1. Cloning and expression of SLAC in *Pichia pastoris*

Low production yield is a major hurdle for application of bacterial laccases at industrial level. Improving production of laccases can therefore enhance their application potential. Laccases from actinobacteria are widely distributed among different *Streptomyces* spp., notably *S. coelicolor* (Arias *et al*., 2003; Endo *et al*., 2003; Lu *et al*., 2013; Machczynski *et al.*, 2004; Molina-Guijarro *et al*., 2009; Niladevi *et al.*, 2008; Suzuki *et al*., 2003). Biotechnological interest in the small laccase produced by *S. coelicolor* has increased in recent years due to its stability under high temperatures and activity within a wide range of pH (Dubé *et al.*, 2008). In the present study, The SLAC gene (ATCC SCO6712) of 1029 nucleotides that translates into a protein of 343 amino acids, was cloned in pPICZαA. The pPICZαA consisted of an AOX1 promoter (for tight regulation), α factor (as a secretion signal) and methanol induced expression of the gene cloned under it. The AOX1 promoter allowed for a high expression level of SLAC in *P. pastoris* by methanol induction. The recombinant plasmid *pPICZαA-SLAC* (**Fig. 4.2**) was confirmed by colony PCR and double digestion with *Eco*RI/*Xba*I **(Fig. 4.3)**. The confirmed construct (*pPICZαA-SLAC*) was transformed into the competent *P. pastoris* GS115 cells. Among 150 clones screened, clone no.128 showed highest enzyme activity and was selected for further investigations. No cell-bound laccase activity was observed, thus showing efficient functioning of secretory signal sequence (α-factor). The transformant, which showed highest activity, was chosen for production of the rSLAC using YP medium.

The produced rSLAC was then investigated for laccase activity using three laccase substrates (ABTS, SGZ and 2,6- DMP). All the substrates were successfully oxidised by the rSLAC.

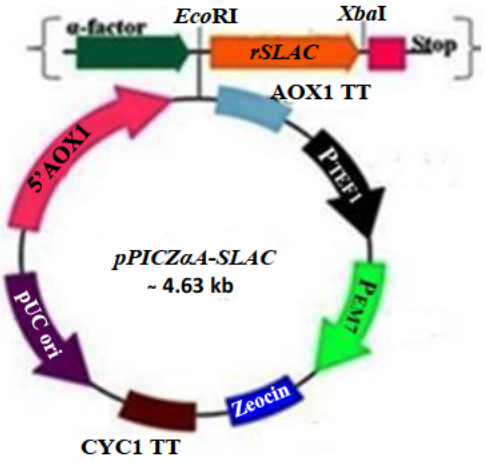


Fig. 4.2 Pictorial representation of *pPICZαA-SLAC* construct for recombinant *P. pastoris*. 5' AOX1, alcohol oxidase (inducible) promoter sequence; α-factor, secretory signal sequence; rSLAC, recombinant small laccase; AOX1 TT, alcohol oxidase transcription termination region; PTEF1, promoter region of transcription elongation factor 1; PEM7, promoter conferring Zeocin resistance; Zeocin, a selectable marker; CYC1 TT, cytochrome c1 transcription termination region; pUC ori, origin of replication.

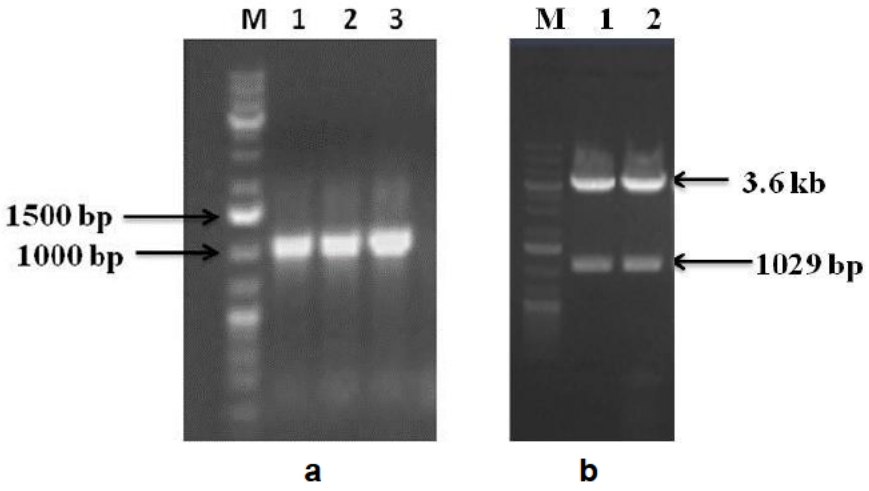


Fig. 4.3 Confirmation of pPICZαA-SLAC construction by (**a**) colony PCR; Lane M- 1 kb plus ladder, Lane 1,2,3- positive clones and (**b**) double digestion; Lane M- 1kb plus ladder, Lane 1,2, double digested pPICZαA-SLAC.

4.3.2 Optimisation of rSLAC production in *Pichia pastoris*

Clone no. 128, harbouring *pPICZαA-SLAC* was cultured to optimise the expression of rSLAC under different medium compositions (YP/BMMY) as well as methanol concentrations. YP medium was found to be the best medium for enzyme production. Of the two media tested, YP medium was found to be more effective in enhancing the rSLAC production. Similar results have been obtained for other recombinant enzymes using *P. pastoris* as an expression host (Ranjan and Satyanarayana, 2016). Thereafter, YP medium was used for further optimisation studies.

Since methanol is a well-known inducer in AOX1-based *Pichia* cultivations (Yurimoto et al., 2002) as well as an important factor in optimisation of recombinant protein production (Sinha *et al.*, 2003), we therefore tested different percentages of methanol to enhance the rSLAC production. Among different concentrations of methanol tested, 2% methanol was found to be the most effective concentration for enhancing rSLAC production; approximately 500 ± 10 U/l was produced (**Fig. 4.4a)**. Reduction in production level after 2% methanol concentration could be due to oxygen depletion which can in turn negatively affect protein expression (Cereghino and Cregg, 2000).

Additionally, maximum production was achieved after 5 days of fermentation, which is significantly higher than other bacterial laccase expressed in *P. pastoris* (Chen *et al*., 2015). However, bacterial CotA laccase expressed in *P. pastoris* achieved maximum production in 12 days, which is possibly due to the use of a different strain of *P. pastoris* (SMD1168H) (Fan *et al.*, 2015). We also studied the laccase activity after preincubation of enzyme at higher temperatures. It is worth noting that the laccase activity increased up to ~7 fold after 30 min incubation at 80 ºC. Similarly, preincubation also enhanced laccase activity in CotA

(Mohammadian *et al.*, 2010) and other thermostable laccases (Hilde *et al.*, 2009). Pre-incubation possibly reduces the time for enzyme to reach the activation energy, which increases the rate of reaction. Pre-incubation could also possibly increase substrate accessibility to active sites through reduction in enzyme aggregation.

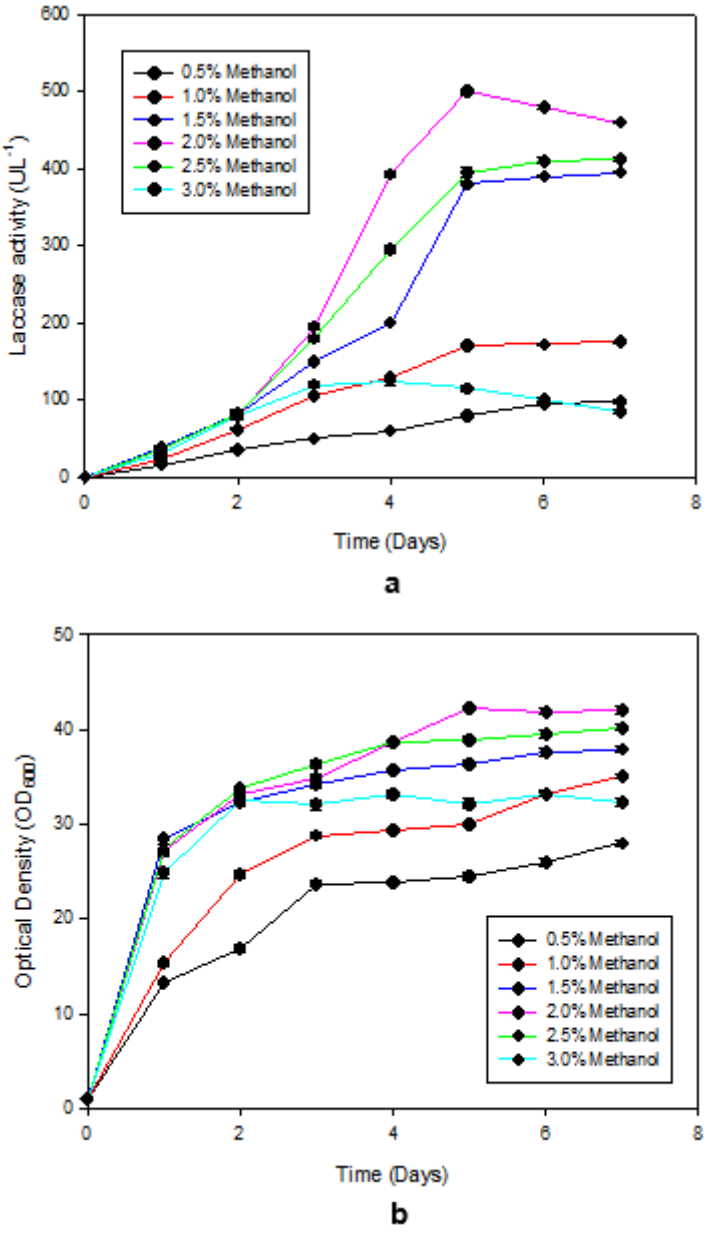


Fig. 4.4 Effect of methanol concentration (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) on **(a)** rSLAC activity and **(b)** cell biomass production of recombinant *P. pastoris* during growth in YP medium. All values are means ± standard deviation.

4.3.3 Purification of rSLAC and determination of molecular mass

The rSLAC was purified to homogeneity and the purity was confirmed by SDS-PAGE. Purification was performed in three steps viz. ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The extracellular rSLAC was purified to 60.35-fold with a specific activity of 8916.66 U/mg (**Table 4.2).** After gel filtration on Sephacryl S-300HR(26/60), the active fraction of SLAC **(Fig. 4.5)** was ran on SDS-PAGE to determine the molecular mass. The purified SLAC appeared as a single band corresponding to a molecular mass of ~38 kDa on SDS-PAGE (**Fig. 4.6**) and was in agreement with the predicted molecular weight derived from the gene sequence (1029 bp).

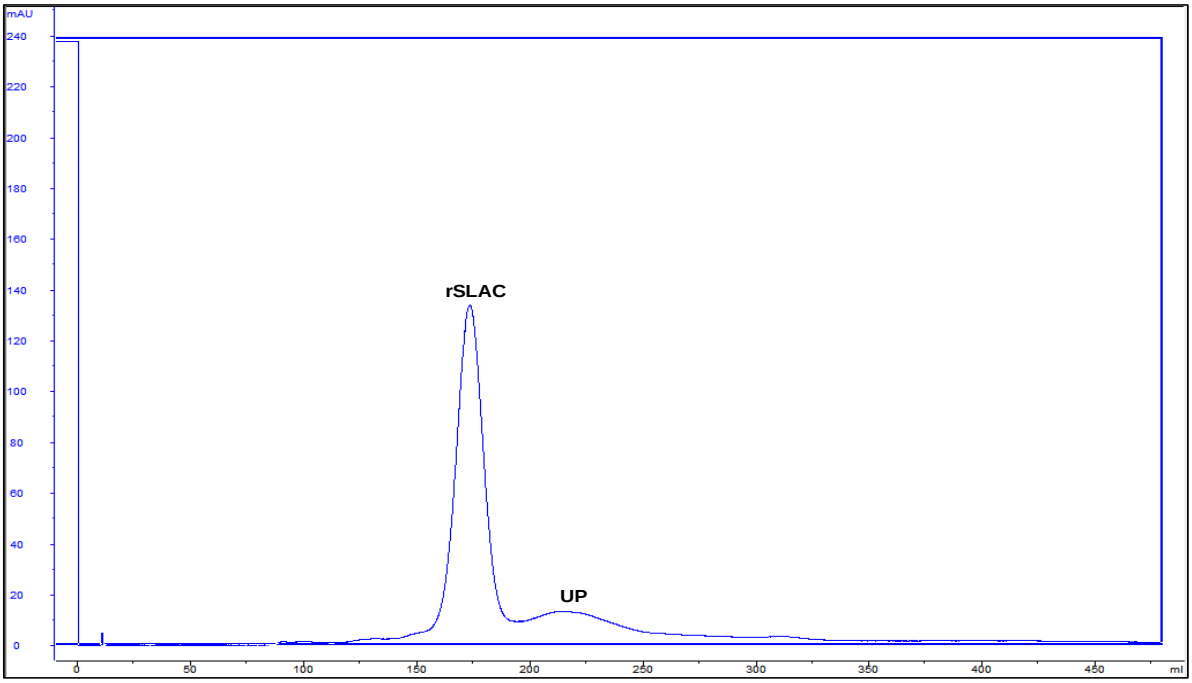


Fig. 4.5 Gel filtration chromatogram of rSLAC using Sephacryl S-300HR(26/60). Eluent: 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl, Fraction size: 1 mL, Flow rate: 0.8 mL/min. rSLAC-purified fraction of laccase and UP-fraction of unknown protein.

Table 4.2 The purification summary of rSLAC produced in *Pichia pastoris*

Purification steps	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Purification fold
Crude enzyme	3.8	0.02572	147.74	1
$(NH_4)_2SO_4$	21.6	0.04309	501.28	3.39
Anion exchange	16.4	0.00295	5,559.32	37.62
Gel filtration	9.63	0.00108	8,916.66	60.35

4.3.4 Purification of rSLAC

The rSLAC was purified to homogeneity and the purity was confirmed by SDS-PAGE. The band corresponded to ~38 kDa on SDS PAGE (**Fig. 4.6**) and was in agreement with the predicted molecular weight derived from the gene sequence (1029 bp). Purification was performed in three steps viz. ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The extracellular rSLAC was purified to 60.35-fold with a specific activity of 8916.66 U/mg (**Table 4.2)**. The size of rSLAC is similar to those of EpoA and SCLAC (Endo *et al.*, 2003; Lu *et al.*, 2013).

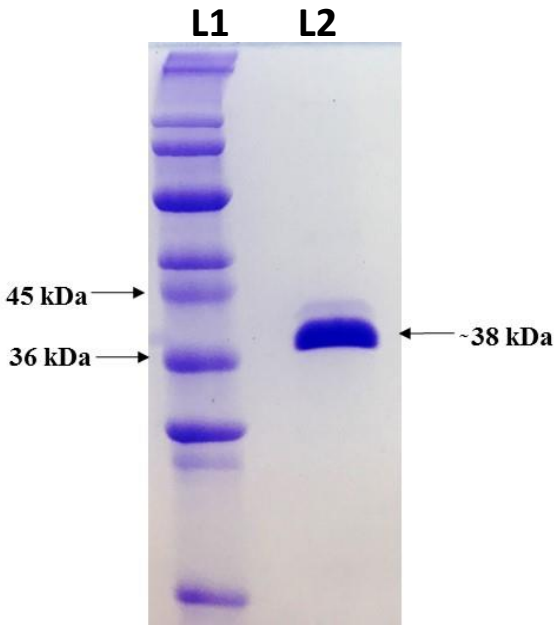


Fig. 4.6 SDS-PAGE analysis of the purified rSLAC: **L1** protein marker; **L2** purified rSLAC

4.3.5 Biochemical characterisation of rSLAC

The pH optima for oxidising ABTS, SGZ and 2,6-DMP was found to be 4.0, 8.0 and 8.0, respectively (**Fig. 4.7a**). The highest activity was observed at 80 ºC with all the substrates (**Fig. 4.7b**).

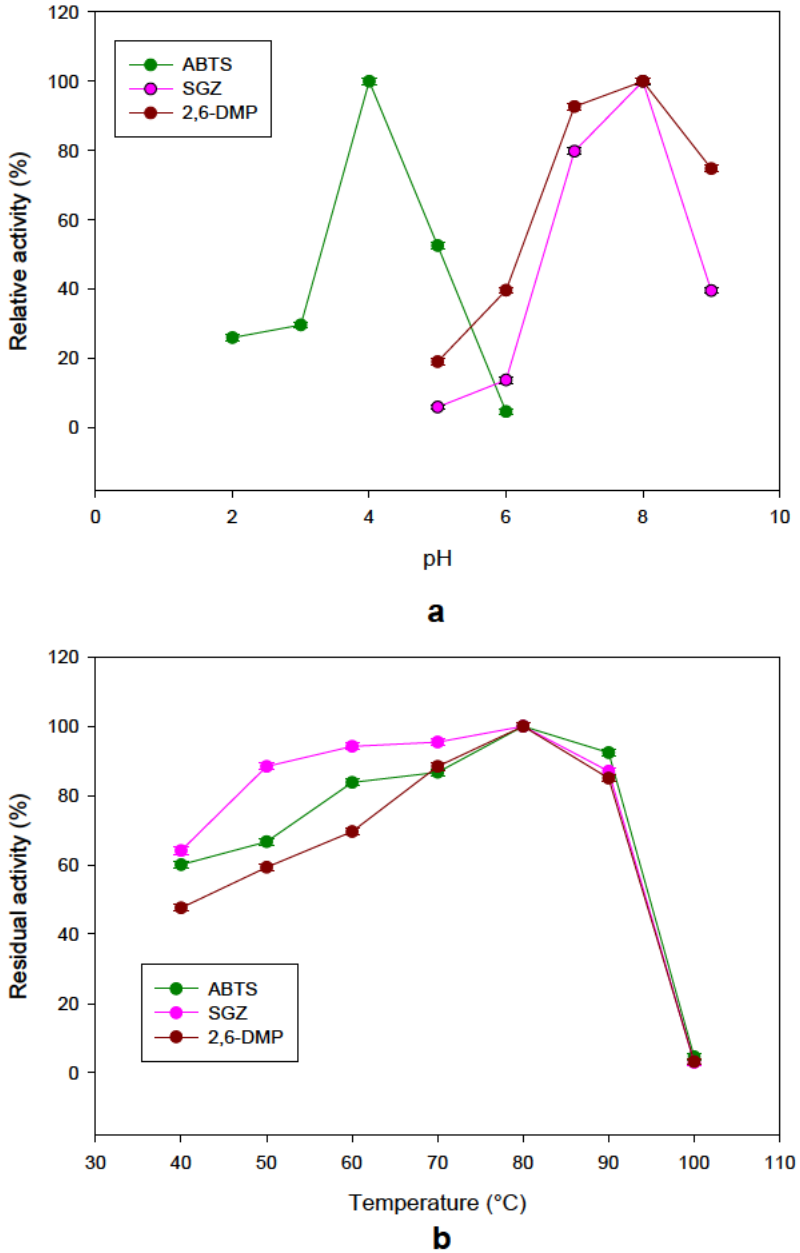


Fig. 4.7 Effect of **(a)** pH on rSLAC activity towards ABTS (1mM), SGZ (0.1 mM), and 2, 6-DMP (2 mM); and (**b)** temperature for laccase activity at optimum pH using ABTS (pH 4), SGZ (pH 8) and 2,6 DMP (pH 8). All assays were done at room temperature (25 ºC).

4.3.6 Kinetic and thermodynamic characterisation of rSLAC

The kinetic constants, K_m values for ABTS, SGZ and 2,6-DMP were 142.85 μM, 10 μM and 54.55 μM with corresponding k_{cat} values of 60.6 s^{-1}, 25.36 s^{-1} and 27.84 s^{-1}, respectively (**Table 4.3**). Additionally, the value of V_{max} and k_{cat}/K_m were calculated for three substrates (ABTS, SGZ and 2,6-DMP) and compared to the other recombinant bacterial laccases (**Table 4.3)**. In the present kinetic study, we observed that K_m values for all substrates (ABTS, SGZ and 2,6-DMP) were lower than other laccases (Durão *et al*., 2008; Fan *et al*., 2015; Martins *et al*., 2002; Wang *et al*., 2015), which indicates that rSLAC has a higher affinity towards the investigated substrates. We also determined the catalytic efficiency and turnover number of rSLAC which was found to be higher than other known bacterial laccases (Chen *et al*., 2015; Fan *et al*., 2015; Wang *et al*., 2015) This indicates that a small amount of rSLAC is potentially more potent in applications (Ranjan *et al*., 2017; Wang *et al*., 2015). This is supported by findings from the present study which showed that only 1 U of rSLAC was efficient in decolourising more than 90% of dyes without the assistance of a mediator. However, 9 U of rCotA was required to decolourise 95% indigo carmine dye in the presence of mediator (Durão *et al*., 2008).

Table 4.3 Kinetic parameters of recombinant bacterial laccases

Recombinant bacterial laccases	**ABTS**			**SGZ**			**2, 6-DMP**			**References**
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m	
rSLAC (*P.pastoris*)	142.85	60.6	0.424	10.86	25.36	2.33	54.55	27.84	0.510	**This study**
SLAC (*E.coli*)	5893	9.49	0.0016	ND	ND	-	5088	8.22	0.001	Prins et al. 2015
rcotA (*P.pastoris*)	146.40	14.4	0.098	12.7	6.9	0.543	ND	ND	-	Wang et al. 2015
rcotA (*P. pastoris*)	162	15	0.092	24	7.6	0.316	166	0.87	0.0058	Fan et al. 2015

ND - not determined. Units: K_m (μM); k_{cat} (s^{-1}); k_{cat}/K_m (s^{-1} μM^{-1})

The activation energy (E_a) for ABTS oxidation by rSLAC was calculated and found to be 38.5 KJ/mol. The deactivation constant (K_d) of rSLAC was calculated from the plot of the $\ln(E_t/E_0)$ versus time (**Fig. 4.8a**). The K_d value was substituted in Eq. (4) to calculate $t_{1/2}$ of the rSLAC. The $t_{1/2}$ values of the rSLAC at 60 ºC, 70 ºC, 80 ºC were found to be 60 h, 32 h and 10 h respectively. Arrhenius plot of the deactivation constant for rSLAC (**Fig. 4.8b**) at different temperatures was plotted to calculate the deactivation energy (E_d), which was found to be 117.275 kJ/mol. First order kinetics was applied on thermal inactivation data to determine the thermodynamic parameters of thermal inactivation of rSLAC. The values ΔG, ΔH and ΔS for thermal inactivation of rSLAC were positive (**Table 4.4**).

Thermostability of an enzyme molecule is defined as its ability to resist thermal inactivation, so higher the value of E_d, the more thermostable is the enzyme. Moreover, thermodynamic parameters also provide a detailed picture of numerous chemical and biological reactions (Samanta et al., 2014). Thermal inactivation of enzyme consists of two steps: Native (N) ↔ Unfolded (U) → Inactive (I), where N stands for native enzyme, U for unfolded inactive enzyme and I for inactivated enzyme (Montes *et al*., 1995). When enzyme is subjected to high temperature, unfolding of native enzymes takes place resulting in the formation of unstable intermediate (U) which could either remain in this state till the input energy is less than the inactivation energy (Kumar and Satyanarayana, 2013) or reversibly refold upon cooling. However, prolonged exposure of heat can lead to crossing of the inactivation barrier, where unfolding occurs and enzyme is permanently inactivated (Das *et al.*, 2012). The higher the value of deactivation energy (E_d), the longer it will be in its native conformation, after being subjected to high temperatures. In the present study, we also found that rSLAC has significantly higher E_d value of 117.275 kJ/mol, which would allow rSLAC to withstand changes to its native conformation when exposed to high temperatures. In addition, we have also calculated

other thermodynamic parameters, such as ΔG, ΔH, and ΔS for the rSLAC. Viability of any chemical reaction is mainly determined by the measurement of change in Gibbs free energy (ΔG), specifically, the transformation of E–S complex into products. The lower the ΔG, the more viable is the reaction. Strikingly, we found that rSLAC has lower ΔG compared to that reported for a fungal laccase (Singha and Panda, 2015). Also, the positive free energy change (ΔG) for the rSLAC indicates the non-spontaneous nature of the thermal denaturation of recombinant enzyme. Besides Gibbs free energy (ΔG), the enthalpy change (ΔH) indicates the energy required for thermal denaturation of protein. The larger the ΔH value, the higher the energy required to break stabilising bonds in the thermal inactivation of the enzyme. Here, we found that rSLAC has higher ΔH value in comparison to the other fungal laccase previously studied (Singha and Panda, 2015). Enzyme denaturation is also accompanied by increase in the disorder of the enzyme structure, measured in terms of entropy change (ΔS), which decreases with increasing enzyme stability. To the best of our knowledge, this is the first report on evaluation of thermodynamic parameters for any known bacterial laccases.

Table 4.4 The thermodynamic parameters of rSLAC measured during thermal deactivation at various temperatures

Temp. (K)	**K_d (h^{-1})**	**$T_{1/2}$ (h)**	**ΔH (KJ/mol)**	**ΔG (KJ/mol)**	**ΔS (J/mol K)**
333.15	0.01155	60	114.505	94.279	60.71
343.15	0.02165	32	114.422	95.392	55.45
353.15	0.0693	10	114.339	94.841	55.2

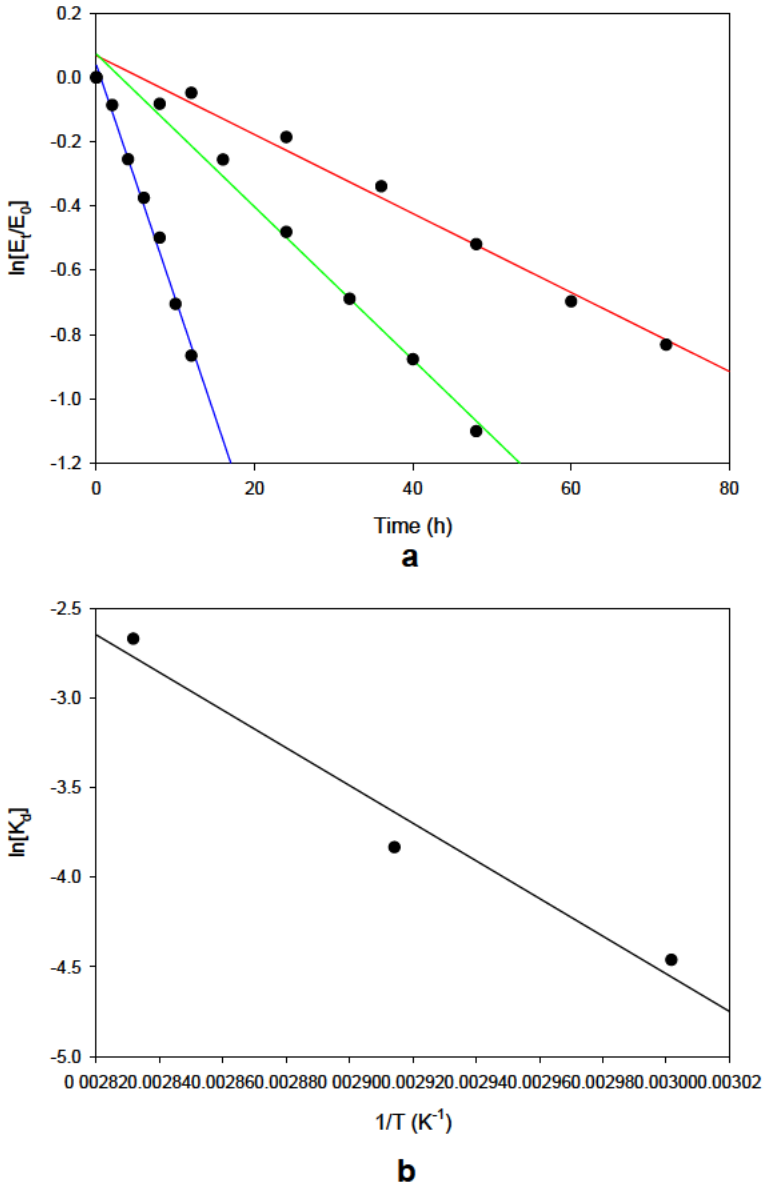


Fig. 4.8 (a) Plot of ln[E_t/E_0] versus time (min) for the calculation of deactivation constant (K_d) and $t_{1/2}$ of rSLAC at different temperatures [60 ˚C (red line), 70 ˚C (green line), 80 ˚C (blue line)]. (**b)** Arrhenius plot of rSLAC for the calculation of deactivation energy (E_d)

4.3.7 Application of rSLAC in dye decolourisation

The purified rSLAC was found to be efficient in decolourising two synthetic dyes belonging to two different classes: triphenylmethane dye (Brilliant Blue G) and a di-azo dye (Trypan

Blue), without the assistance of a mediator. Maximum dye decolourisation was achieved within 6 h (**Fig. 4.9a**) and the dyes were better decolourised in acidic than alkaline pH (**Fig. 4.9b**). Approximately 92.2% of BBG and 91.7% of TB were decolourised (pH 4) by the rSLAC without the aid of a mediator compared to 86.2 % of BBG and 46.7% TB at pH 8.0 (**Fig. 4.9b**). In the control reaction, no decolourisation was observed indicating the catalytic role of rSLAC in dye decolourisation. However, in earlier reports, SLAC decolourised only 8% of an Indigo dye without the assistance of a mediator (Dubé *et al*., 2008) Among different pH (4, 6 and 8) tested we did not observe any significant effect of pH on decolourisation of Brilliant Blue G dye. This indicates that rSLAC remains efficient in acidic to alkaline range of pH. However, there was decrease in decolourisation of Trypan Blue (azo dye) in alkaline pH. Similarly, alkaline pH had negative effect on azo dye decolourisation by other laccases (Ashrafi *et al*., 2013; Mirzadeh *et al*., 2014). It is worth mentioning the recalcitrant nature of the azo group of dyes and the resistance to laccase-catalysed oxidation increases with increase in the number of azo groups (Ranjan *et al*., 2017). Usually, decolourisation of azo dyes requires redox (Camarero *et al*., 2005; Forootanfar *et al.*, 2016; Ostadhadi-dehkordi *et al*., 2012; Ranjan *et al.*, 2017), however, rSLAC was able to decolorise TB (azo dye) without the aid of a redox mediator (Giardina et al., 2010), which is consistent with findings for CotA laccase (Pereira *et al*., 2009). This suggests that bacterial laccases have potential for decolourising recalcitrant dyes used in textile industries.

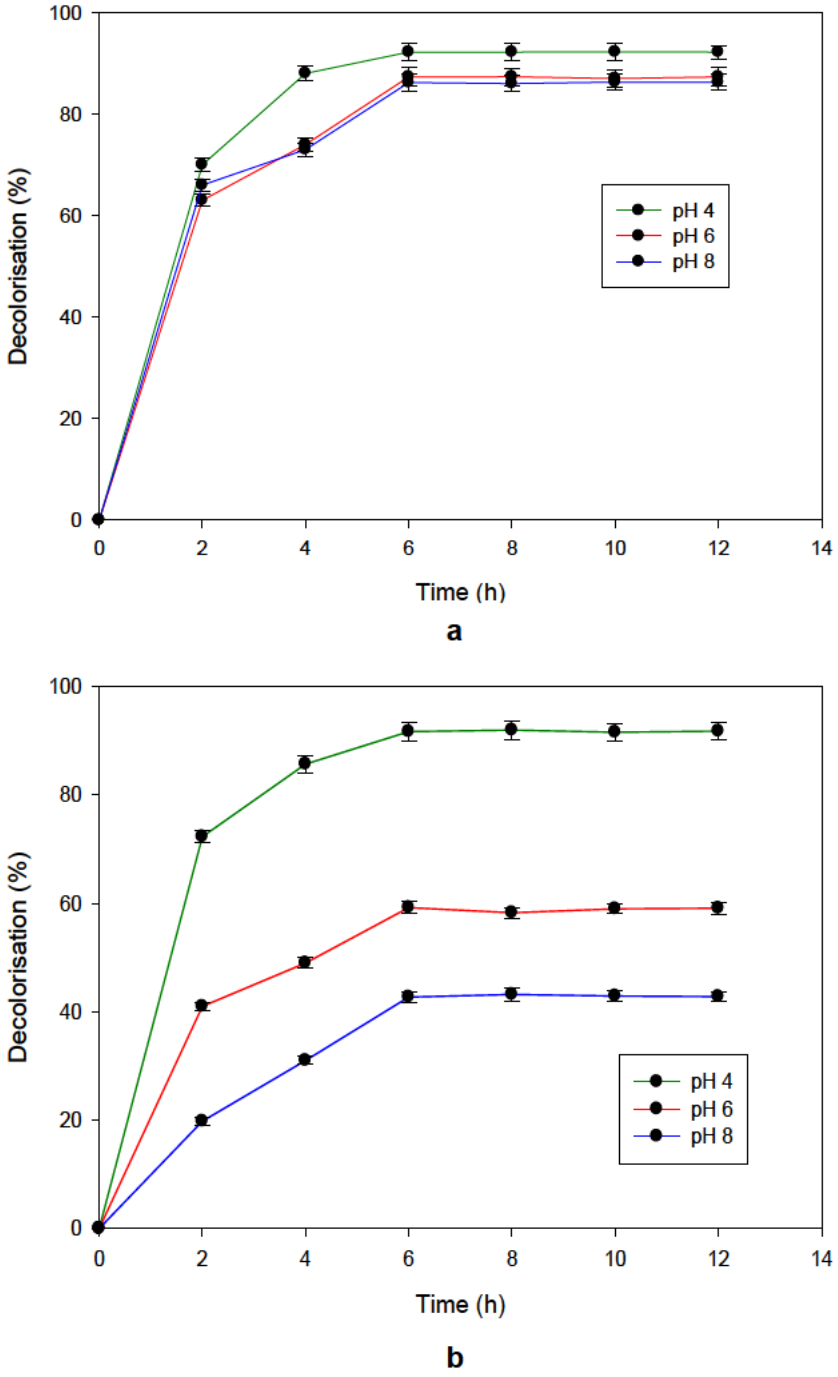


Fig. 4.9 Effect of time and pH on dye decolourisation by purified rSLAC: (**a**) Brilliant Blue G and (**b**) Trypan blue. All values are means ± standard deviation.

4.4 Conclusions

SLAC from *S. coelicolor* was functionally expressed and secreted in *P. pastoris*. Optimisation of production medium and methanol concentration enhanced rSLAC production. The rSLAC showed good decolourisation potential towards Brilliant Blue G and Trypan Blue. Extracellular expression of SLAC in *P. pastoris* opens up avenues for its wide application in various industries.

CHAPTER 5

Enhancing the expression of recombinant small laccase (rSLAC) in *Pichia pastoris* by a double promoter system and its application in antibiotics degradation

Abstract

Low expression levels remains a challenge in the quest to use the small laccase (rSLAC) as a viable catalyst. In this study, a recombinant *Pichia* strain (rSLAC-GAP-AOX producing rSLAC under both AOX and GAP promoters) was generated and cultivated in the presence of methanol and mixed feed (methanol:glycerol). Induction with methanol resulted in a maximum laccase activity of 1200 U/L for rSLAC-GAP-AOX which was approximately 2.4-fold higher than rSLAC-AOX and 5.1-fold higher than rSLAC-GAP. The addition of methanol:glycerol in a stoichometric ratio of 9:1 consistently improved biomass and led to a 1.5-fold increase in rSLAC production as compared to induction with methanol alone. The rSLAC removed 95% of 5 mg/L ciprofloxacin and 99% of 100 mg/L tetracycline in the presence of a mediator. Removal of TC resulted in complete elimination of antibacterial activity while up to 48% eduction in antibacterial activity was observed when CIP was removed.

5.1 Introduction

Laccases are regarded as versatile biocatalysts owing to their broad substrate specificity enabling potential use in a wide range of applications (Xia *et al*., 2019). Bacterial laccases could prove to be useful alternatives to fungal laccases due to their intrinsic ability to tolerate high temperature and alkaline pH (Yadav *et al*., 2018). However, low production yield from native sources could present a limitation on potential industrial application (Dubé *et al*., 2008). Production of laccases in recombinant hosts such as *Pichia pastoris* could overcome this obstacle. Selection of suitable strong promoters as well as the signal sequence can be made during recombinant protein production which could direct the protein to culture media, thereby simplifying enzyme purification. Furthermore, optimising the expression conditions such as pH or temperature could also increase the enzyme yield (Antošová and Sychrová, 2016).

Pichia pastoris, a methylotrophic yeast is an excellent host in producing functionally active recombinant proteins due to its excellent protein secretory capability (Looser *et al*., 2015), ability to attain high cell densities using minimal media, stable integration of the gene of interest (GOI)(Wu *et al*., 2003), low secretion of native proteins and availability of strong promoters (Yadav *et al*., 2018). AOX1 and GAP promoter-based systems are two of the easy to use expression systems commercially available for *P. pastoris*, where the former is methanol-inducible and the latter, constitutively expressed (Gidijala *et al*., 2018). AOX1 is the most preferred promoter system due to the strong and tight regulation of the GOI cloned under its control and this has allowed efficient production of several industrial enzymes (Öztürk *et al*., 2017). In addition, growth phase can be decoupled from protein production phase in the methanol inducible expression systems (Gidijala *et al*., 2018). Furthermore, strategies have been adopted to enhance the production level in P_{AOX1}-based systems such as codon

optimisation, multi-copy gene insertion, process optimisation and co-expressing folding or secretion factors (Cámara *et al*., 2017).

In our previous studies, the small laccase from *Streptomyces coelicolor* was heterologously expressed in *P. pastoris* under AOX promoter (Yadav *et al*., 2018). However, the slow growth rate and low enzyme productivity (mainly due to the use of methanol as both inducer and carbon source) is a bottleneck for effective enzyme production (Parashar and Satyanarayana, 2016). The AOX-based promoter system for heterologous protein expression in *P. pastoris* involves three phases, namely (i) glycerol batch phase, (ii) glycerol fed batch phase and (iii) methanol induction phase. However, the first two phases comprises of only biomass generation and the final phase (methanol induction phase) is involved in the expression of recombinant protein (Cunha *et al*., 2004; Hellwig *et al*., 2001; Holmes *et al*., 2009). The use of a dual promoter system i.e., constitutive and inducible promoter, in a single host is an alternative to enhance the heterologous enzyme production during all three phases (Parashar and Satyanarayana, 2016).

Therefore, the present investigation aims to enhance the extracellular expression of a recombinant small laccase (rSLAC) in *P. pastoris* by integrating the small laccase gene under the control of both constitutive and inducible promoters in a single clone. We also investigated the effect of different parameters on laccase production *viz*., pH, temperature and co-feeding with methanol:glycerol at shake flask level (50 ml). In addition, the purified rSLAC was applied in the degradation of two broad spectrum antibiotics, ciprofloxacin (CIP) and tetracycline (TC), as a model of their remediation from contaminated environments.

5.2 Materials and methods

5.2.1 Strains, vectors and chemicals

Escherichia coli DH5α and *P. pastoris* GS115 (Invitrogen) were used as the hosts for cloning and expression of laccase, respectively. *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used to determine antibacterial activity and were obtained from the Cape Peninsula University of Technology, South Africa and are currently deposited in the stock culture collection in the Department of Biotechnology and Food Technology, Durban University of Technology. Plasmids, pPICZαA and pGAPZαA (Invitrogen), were used for the expression of laccase.

Yeast extract peptone dextrose (YPD), yeast extract peptone (YP) were used to culture the methylotrophic yeast, *P. pastoris* and were prepared according to the manual of Easy select Pichia expression kit (Invitrogen). pET28a (+) (Novagen) was used as a source of the kanamycin resistance gene used in this study. CIP, TC, 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and G-418 were purchased from Sigma Aldrich. Stock solutions of CIP (0.1 mg/ml) and of TC (0.2 mg/ml) were prepared and stored at 4°C. The restriction enzymes (*Eco*RI, *Nco*I, *Nde*I, *Sac*I, *Avr*II and *Xba*I) were procured from New England Biolabs and primers (**Table 5.1**) from Integrated DNA Technologies (IDT).

5.2.2 Construction of pGAPKαA vector with unique selection marker

Primers **P1** (pGAPαA_F) and **P2** (pGAPαA_R) (**Table 5.1)** with flanking regions of an *Nco*I and an *Nde*I restriction sites, respectively, were used to amplify pGAPZαA vector lacking zeocin encoding region. A unique *Nde*I site was inserted in pGAPZαA to clone in the kanamycin resistance encoding region. Kanamycin resistance encoding sequence (816 bp) was amplified using primers **P3** (Kan_F) and **P4** (Kan_R) from pET28a (+) vector, allowing for the

introduction of *Nco*I and *Nde*I restriction sites, respectively. All amplifications were carried out in a GeneAmp PCR System 9700, Applied Biosystems using Phusion High-Fidelity DNA Polymerase in a 50 μL reaction mixture under the following PCR conditions: denaturation at 98°C for 1 min; 30 cycles of denaturation at 98°C for 30 s, annealing at 58°C for 30 s, elongation 72°C for 6 min; and a final elongation at 72°C for 10 min. PCR products were then double digested with *Nco*I and *Nde*I and ligated to generate vector pGAPKαA. The ligation mix was transformed into *E. coli* DH5α competent cells and screened for positive clones by colony PCR. Ligation and colony PCR were carried out using standard protocols.

Table 5.1 Primers used in this study

Primers	Oligonucleotide sequence (5'→ 3')	Product
P1	TCTACCATGGTTTAGTTCCTCACCTTGTCGTA	Vector pGAPZαA devoid of zeocin region
P2	AACGCATATGACGTCCGACGGCGGCCCACGGGT	
P3 (Kan_F)	CTAAACCATGGGCCATATTCAACGGG	Gene encoding for kanamycin resistance
P4 (Kan_R)	TTCCACATATGTTAGAAAAACTCATCGAG	
P5	CGGAATTCATGGACAGGCGAGGCTTTAA	Encoding region of SLAC
P6	CGTCTAGATAA GTGCTCGTGTTCGTGTGCGG	

5.2.3 Construction of pGAPKαA-*SLAC*

Primers (**P5** and **P6, Table 5.1**) and conditions used for the amplification of the *SLAC* gene were used as previously described (Yadav *et al*., 2018). pGAPZαA vector was modified to pGAPKαA according to Parashar and Satyanarayana (2016), whereby the gene conferring zeocin resistance was replaced by the kanamycin resistance gene (816 bp). Restriction

digestion of the PCR product (SLAC) and pGAPKαA were carried out with *Eco*RI and *Xba*I for 1 h at 37°C in the presence of Cut Smart buffer and the products purified by GeneJet purification Kit (Thermofischer Scientific). Ligation of digested products was carried out for the generation of pGAPKαA-*SLAC* (**Fig. 5.1a**) and pPICZαA-*SLAC* (**Fig. 5.1b**) as previously described (Yadav *et al.*, 2018). The ligation mixture was transformed into *E. coli* DH5α competent cells and the transformants obtained were confirmed by colony PCR and double digestion, as well as followed by automated DNA sequencing.

5.2.4 Transformation into *P. pastoris*

The electrocompetent *P. pastoris* cells were prepared and confirmed constructs were transformed according to the manufacturer's protocol (Invitrogen). *SLAC*-GAP was generated by transforming wild type *P. pastoris* cells with linearised pGAPKαA-*SLAC*. The transformed cells were plated onto YPD agar (g/l): yeast extract 10, peptone 20 and dextrose 20, agar 20, containing varying concentrations (200 - 400 µg/ml) of G-418 (an aminoglycoside antibiotic) and grown at 30°C for 72 h. Since the ability of the transformants to tolerate high concentration of antibiotic correlates with the gene copy number. Clones capable of growing at the highest concentrations of geneticin were selected for shake flask screening. In order to identify the positive transformants, colony PCR was performed. Positive clones were then screened for the best producer of laccase by conducting small scale expression trials. The transformants were grown in YPD broth (50 ml) followed by separation of culture supernatant from the cell biomass by centrifugation (1500 x g, 10 min). Thereafter, 1 mM $CuSO_4$ was added to the supernatant and a quantitative laccase assay was performed. The best clone shortlisted from screening had laccase activity of 234 U/L. Further, to generate *SLAC*-GAP-AOX strain, pGAPKαA-*SLAC* were made competent and transformed by pPICZαA-*SLAC* (linearised by *Avr*II) vector. The transformed cells were plated on YPD agar containing zeocin and grown at

30°C for 72 h. Isolated resistant colonies were then grown in YP broth along with induction by 0.5% (v/v) methanol. Supernatants were harvested after 5 days of cultivation and assayed for laccase activity. Strains exhibiting the highest activity were shortlisted for further investigation.

5.2.5 Determination of laccase activity

Laccase activity was determined using ABTS as a substrate, as described previously (Yadav *et al*., 2018). Oxidation of ABTS (1 mM) was measured at 420 nm ($\varepsilon = 36{,}000 M^{-1}\ cm^{-1}$) in 20 mM acetate buffer (pH 4.0). One unit of laccase activity (U) was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute (Jin *et al*., 2016).

5.2.6 rSLAC production and optimisation in shake flasks

For expression under the *GAP* promoter, a single colony was inoculated in 10 mL of YPD medium in a 250 mL flask and the culture was grown at 30°C and 250 rpm until the culture reached an OD_{600} of 2-6 (in about 16-18 h). Then the fresh (50 mL) YPD medium was inoculated with 1% (v/v) seed culture and incubated at 30°C and 250 rpm for 7 days. Aliquots were drawn every 24 h for enzyme assays. For induced expression under *AOX* promoter, cells were harvested from a 24 h old primary culture by centrifuging at 1500 g for 5 min and resuspended in YP medium till OD_{600} reached 1.0. Methanol was added to a final concentration of 2% (v/v) after every 24 h interval to maintain induction, and samples were collected after every 24 h to analyse for laccase activity. Optimum temperature was determined by growing the recombinant strains at different cultivation temperatures (16, 20, 24, 28 and 32°C) and the optimum pH for laccase production was determined over the pH range pH 4.0-8.0. Experiments were carried out in triplicate and results are shown as the mean ± standard deviation (SD) of three independent experiments.

5.2.7 Optimisation for mixed fed batch fermentation in shake flasks

The influence of a mixed feed (glycerol:methanol) was investigated in the production of rSLAC by the *Pichia* strain harbouring rSLAC-AOX-GAP and rSLAC-AOX. A mixture of methanol and glyceroll was added after every 24 h in different stoichometric concentrations (100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100) to a final concentration of 2% (v/v) in order to maintain induction. Sampling was performed every day to determine cell growth (OD_{600}) and the expression level of rSLAC.

5.2.8 Degradation and detoxification of CIP and TC by rSLAC

5.2.8.1 Enzymatic transformation of CIP and TC in aqueous solution

For preliminary set of experiments, the reaction mixture (1ml) consisted of individual antibiotics (CIP at 5 mg/L; TC at 50 mg/L) in Tris-HCl buffer (50 mM, pH 8.0), and acetosyringone (AS) (4 mM). The reaction was started by adding rSLAC (0.4 U) and was incubated in the dark with mild shaking (50 rpm) at 37°C. A parallel control reaction was prepared that consisted of antibiotic with mediator to rule out the degradation of antibiotic by abiotic factors.

Optimisation of reaction conditions in enzyme catalysed systems can accelerate the degradation efficiency of antibiotics. Therefore, to examine the optimum conditions for degradation of model antibiotics (TC and CIP) by rSLAC, we investigated degradation of different initial concentrations of antibiotics (CIP: 2.5-10 mg/L; TC: 50-200 mg/L), at various pH levels (4.0-8.0) and AS concentrations (1-4 mM).

For time course studies, reactions were carried out under optimum conditions and at pre-selected time intervals, samples were withdrawn and stored at -20 ºC until analysis. The

residual concentration of CIP and TC in the reaction mixtures were analysed by HPLC as described in **5.2.8.2**.

Removal efficiency was calculated according to Sun, Huang and Li (2017) as follows:

$$\text{Removal \%} = (C_0 - C_t)/C_0 * 100$$

where, C_0 (mg/L) corresponds to the concentration of antibiotic at time 0 and C_t (mg/L) corresponds to the residual concentration (after rSLAC treatment) of antibiotic at time t.

5.2.8.2 Quantitative analysis of antibiotics and its degradation by HPLC

Antibiotics and their degradation products were analysed using reversed phase chromatography on a Shimadzu HPLC system from Dionex (Massachusetts, USA) equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Chromatographic separation was achieved on a C18 column (Sunfire, Waters). The mobile phases used for analysis were 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.5 mL/min. The injection volume was 10 µl and an oven temperature of 25°C was used. Isocratic elution was used with 15% B for 30 min and the degradation products were quantified at 254 nm.

5.2.8.3 Assay for bacterial growth inhibition

The residual antibacterial activities of the degradation products of CIP and TC (after rSLAC treatment) were evaluated as percentage growth inhibition or qualitatively using well diffusion assays.

A well diffusion method was employed to determine the antibacterial activity of the samples according to Pan *et al.* (2018) with slight modifications. A Gram positive (*S. aureus,* ATCC 29213) and a Gram negative (*E. coli*, ATCC 25922) bacterial strains were selected as representative microorganisms for this study. Briefly, 0.1 mL of each culture (OD_{600} of 2.0) was spread onto nutrient agar plates. Thereafter, wells were punctured into the agar surface using a sterile well borer. Each well was spiked with 40 µL of treated samples followed by an overnight incubation at 37°C. Two set of controls were set-up where the first control consisted of the untreated antibiotic solution (2.5 mg/L of CIP and 100 mg/L of TC), while the second set of controls consisted of solvents used for preparing stock solutions of CIP and TC, i.e. double-distilled water (for TC) and DMSO (for CIP), to rule out the effect of solvent. The zone of inhibition (indicating antibacterial activity) was measured in millimetres and each experiment was repeated in triplicate; the mean values ± SD are reported.

Growth inhibition tests were also conducted using *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 as previously described by Sun, Huang and Li (2017) with minor modifications. Briefly, the strains were cultured in nutrient broth (NB) at 37°C with shaking at 200 rpm for 24 h. Subsequently, standard antibiotic solutions (2.5 mg/L of CIP and 100 mg/L of TC) and the treated reaction mixtures of CIP and TC (40 µL) were added into the NB medium along with the bacterial suspension (100 µL) and incubated under the same conditions. After 24 h incubation, absorbance of the samples was measured which were then converted into percent growth inhibition according to the equation:

$$\text{Growth inhibition \%} = (OD_{600C} - OD_{600T})/OD_{600C} \times 100,$$

Where OD_{600C} corresponds to the OD of the control sample (i.e. *E. coli* or *S. aureus* culture) and OD_{600T} corresponds to the OD of the test sample consisting of the rSLAC treated solutions of CIP and TC.

5.3 Results and discussion

5.3.1 Development of recombinant *Pichia* strain with dual promoter for heterologous production of rSLAC

To accomplish this, the vector pGAPZαA was modified by incorporating the Kanamycin resistance gene **(Fig. 5.1)**. The engineered pGAPZαA, now lacks zeocin encoding region and confers resistance against kanamycin in bacteria as well as geneticin in yeast. Two *Pichia* strains were generated: pPICZαA-*SLAC* and pGAPKαA-*SLAC* producing SLAC under AOX and GAP promoters, respectively. Furthermore, the recombinant *Pichia* strain harbouring pGAPKαA-*SLAC* were made competent (by electroporation) and transformed for the second time with linearised pPICZαA-*SLAC* to generate rSLAC-GAP-AOX capable of synthesizing SLAC in the presence of both methanol and glycerol. This clone showed resistance towards both the marker genes (G-418 and zeocin), indicating successful integration of both plasmids.

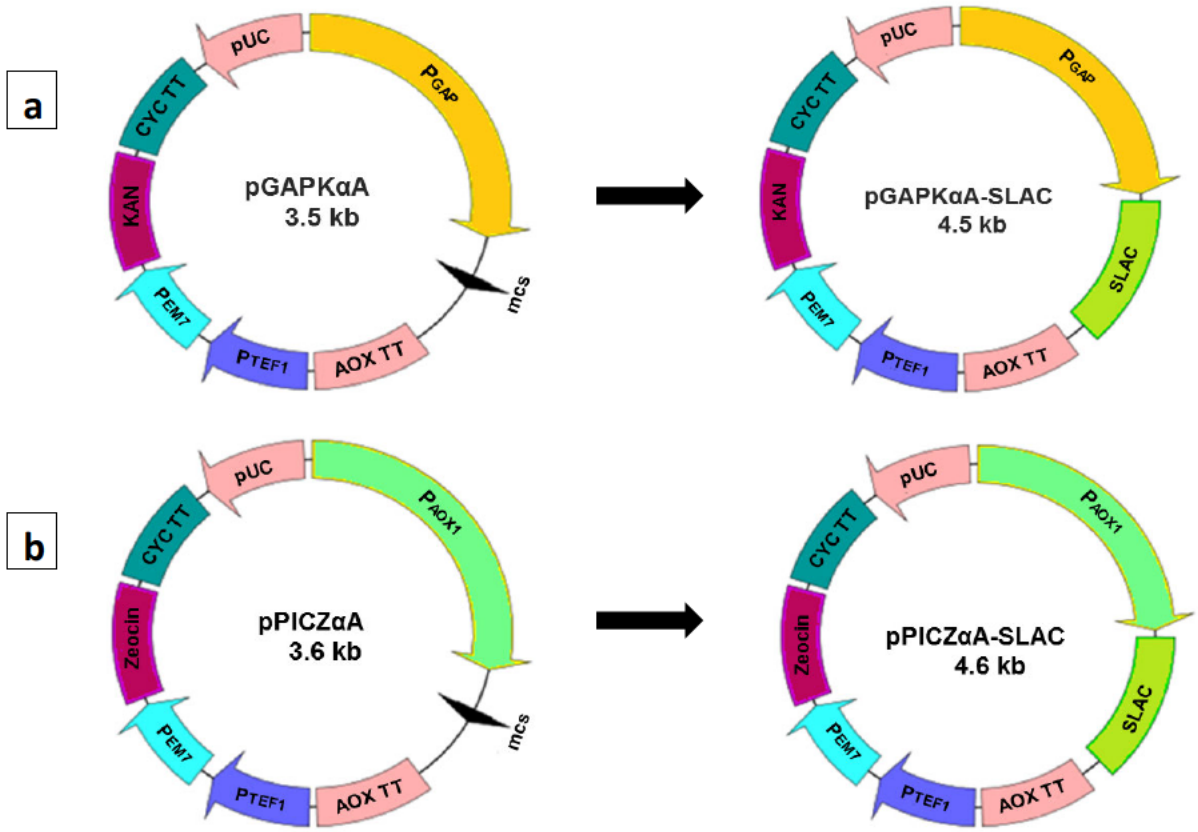


Fig. 5.1 Strategy used for the construction of **(a)** pGAPKαA-SLAC and **(b)** pPICZαA-SLAC with different antibiotic selection markers. These vectors drive the expression of the small laccase (rSLAC) from *Streptomyces coelicolor* in *P. pastoris*.

5.3.2 rSLAC production by recombinant *Pichia pastoris* (rSLAC-GAP-AOX) strain in shake flask cultures

The profile for cell growth and rSLAC production from the recombinant *Pichia* strains harbouring single and double promoters is shown in **Fig. 5.2.** Maximum rSLAC expression level was achieved after the 5th day of cultivation (1200 U/L) which was 2.4-fold higher than the single promoter system. In addition, there was also an increase in the cell growth of the recombinant *Pichia* strain with rSLAC-GAP-AOX (**Fig. 5.2**).

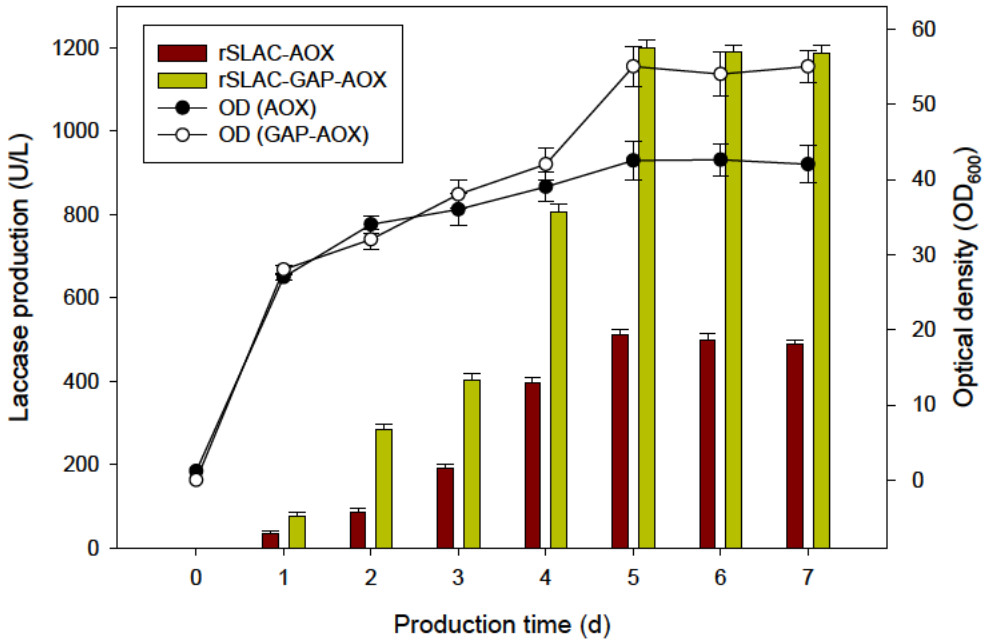


Fig. 5.2 Time course of cell growth and rSLAC production by the recombinant *Pichia* strain harbouring rSLAC-AOX-GAP or rSLAC-AOX. The recombinant *Pichia* strain was cultivated in YP medium and was supplemented with 2% (v/v) methanol after every 24 hours. Data are presented as the mean ± standard deviation (SD) of triplicate determinations.

5.3.3. Optimisation of cultivation parameters

5.3.3.1 pH and temperature

P. pastoris can tolerate a broad range of pH and therefore the expression of rSLAC was evaluated over a pH range of 4.0 to 8.0. Although *P. pastoris* growth was not greatly affected by pH, laccase expression increased with an increase in the pH of the medium (up to pH 7.0) **(Fig 5.3a)**. The results also indicate the stability of rSLAC over a broad pH range. However, low activity at acidic pH (i.e. pH 4.0) can be attributed to the activation of acidic proteases which could lead to the loss of enzyme stability. This is consistent with other reports which

also documented the proteolytic degradation of enzymes at low pH (Inan *et al*., 1999; Soden *et al*., 2002). In addition, it is worth mentioning that optimal pH of the medium can vary for different recombinant proteins.

Laccase expression generally increased with increase in cultivation temperature **(Fig. 5.3b).** An optimum temperature of 28°C was recorded for both rSLAC-AOX and rSLAC-GAP-AOX strains; a slightly lower expression level was observed at 32°C. After 5 days of cultivation, rSLAC production level at 28°C was approximately 3.2 times higher than at 16°C. Clearly, lowering the cultivation temperature did not favour the expression of rSLAC in *P. pastoris* which could be ascribed to the low biomass at 16°C **(data not shown)**.

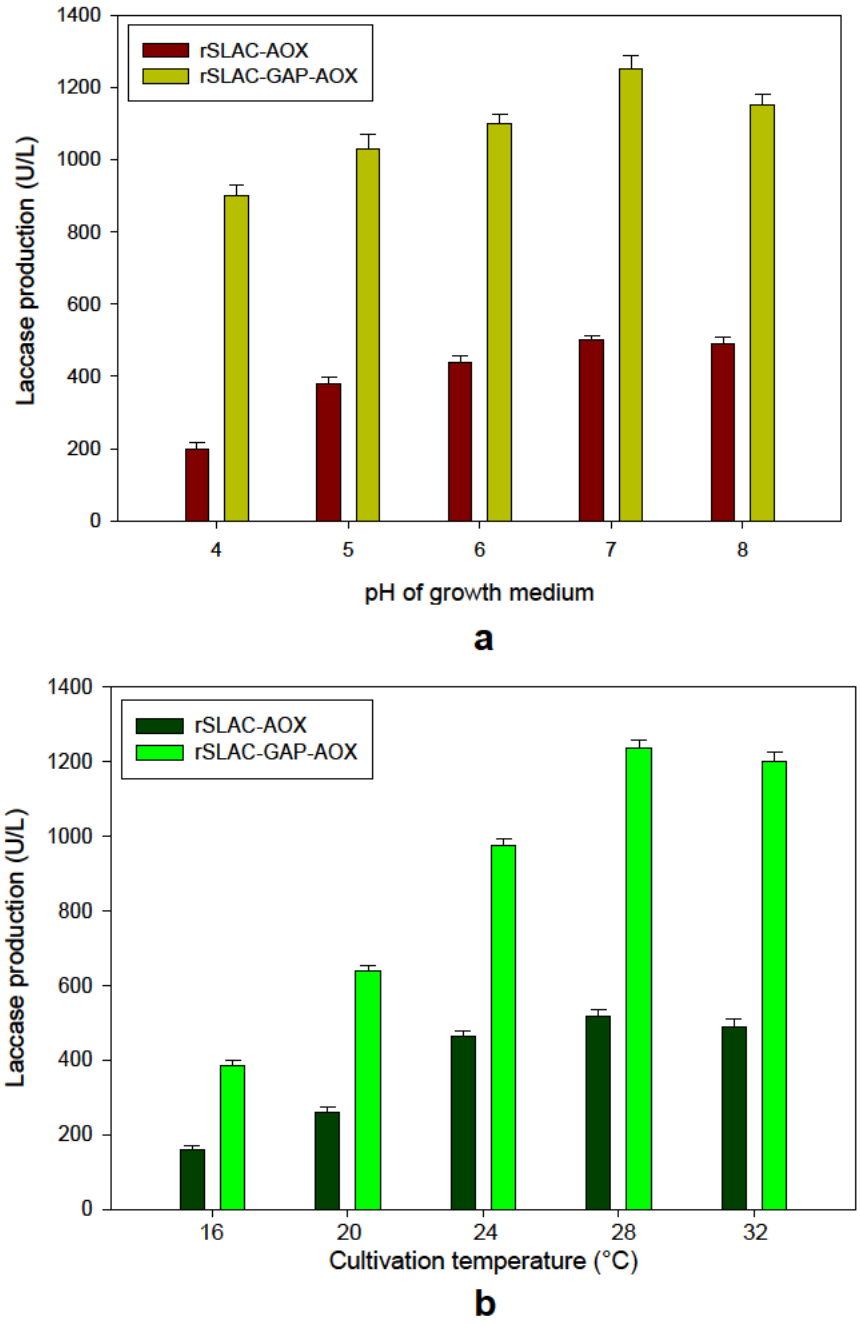


Fig. 5.3 Optimisation of **(a)** the pH of the medium used and **(b)** cultivation temperature for the production of laccase from rSLAC-AOX-GAP and rSLAC-AOX *Pichia* strains, respectively. The results are represented as an average value of three separate experiments ± SD.

5.3.3.2 Mixed feed

The rSLAC-GAP-AOX strain produced higher titres of laccase when the production medium (YP) was supplemented with a mixed feed of methanol:glycerol as compared to rSLAC-AOX. Among different ratios of methanol:glycerol tested, maximum production level was achieved with a 9:1 ratio of methanol:glycerol **(Fig. 5.4a)**. However, in case of the strain harbouring rSLAC-AOX, maximum enzyme production was obtained with 100% methanol induction. When a single promoter system was supplied with a mixed feed, laccase production was negatively affected **(Fig. 5.4b)**. This is possibly due to the inability of this system to utilise glycerol, which in turn represses the AOX promoter (Parashar and Satyanarayana, 2016) resulting in repression of rSLAC transcription.

Even though methanol can be utilised by *P. pastoris* as a sole energy source (Parashar and Satyanarayana, 2016), excessive methanol can negatively impact cell growth due to the accumulation of toxic products of methanol oxidation such as formaldehyde and hydrogen peroxide (Zhang *et al*., 2003). Therefore, the growth rate of *Pichia* cells is lower in methanol as compared to glycerol. However, low enthalpy of combustion of glycerol leads to lower heat production and low oxygen consumption rate during growth (Parashar and Satyanarayana, 2016). In high cell density fermentation with *P. pastoris*, maintenance of temperature and adequate oxygen supply represent technical limitations. Therefore, in such a case, supplementing with methanol:glycerol could enhance cell biomass and process productivity (Parashar and Satyanarayana, 2016). Several authors have reported positive influence of mixed feed of glycerol and methanol on cell culture viability and protein production rate (McGrew *et al*., 1997; Parashar and Satyanarayana, 2016; Woo *et al*., 2004). It is evident from the present study, that the mixed feed positively influenced cell biomass production which, however, is

not consistent with rSLAC production **(Fig. 5.4a & 5.4b)**. This could be attributed to the repression of AOX promoter by residual glycerol (Cereghino *et al*., 2002).

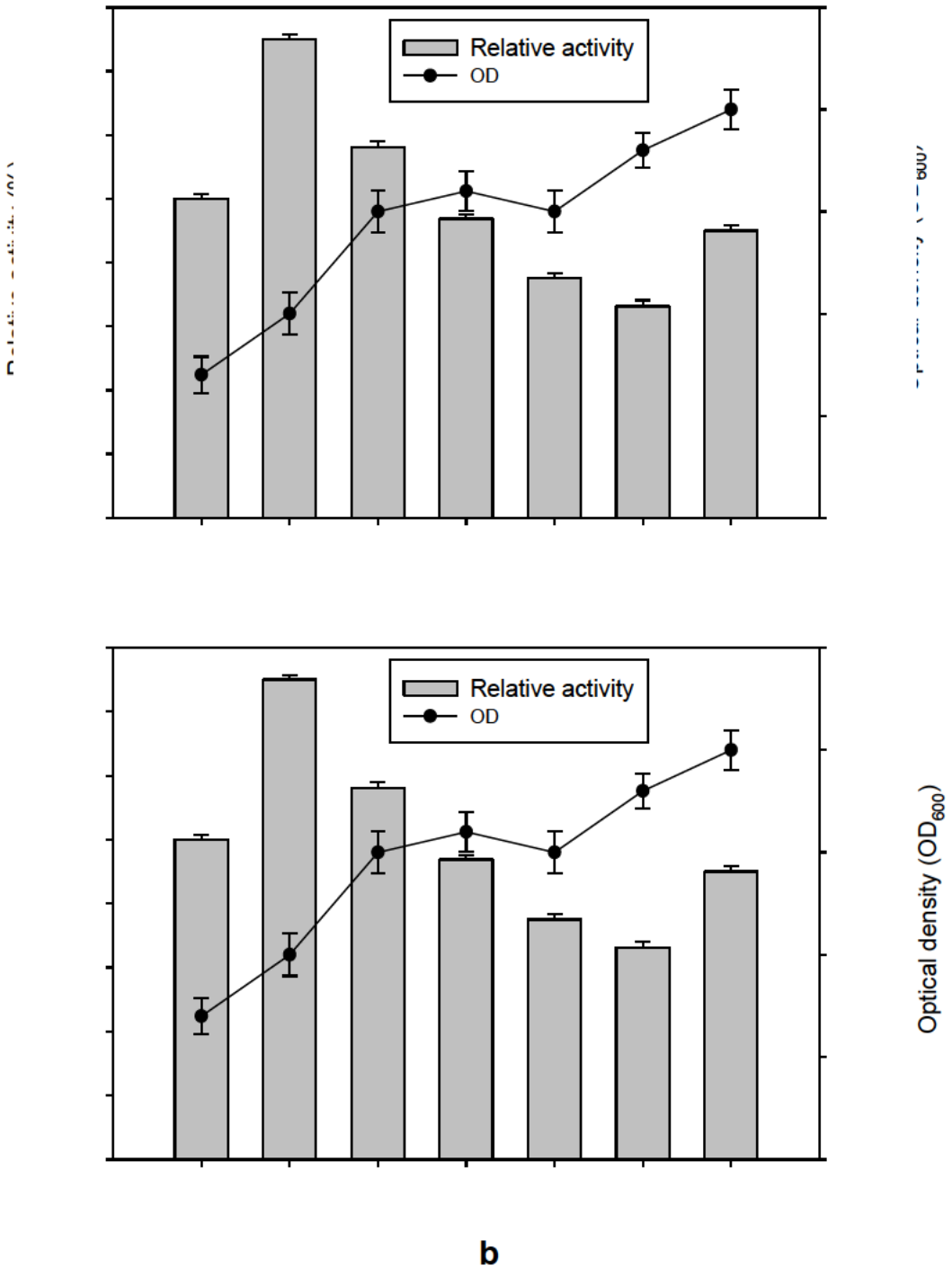


Fig. 5.4 Effect of mixed feed cultivation on cell biomass (-▬-) and rSLAC expression by a *Pichia* strain harbouring **(a)** rSLAC-AOX-GAP and **(b)** rSLAC-AOX, cultivated in YP

medium. A, B, C, D, E, F and G denotes the 2% (v/v) mixed feed of methanol:glycerol [100:0, 90:10, 80:20, 70:30, 60:40, 50:50 and 0:100, respectively]. Data are presented as means ± SD (n=3).

5.3.4 Degradation and detoxification of antibiotics

5.3.4.1 Degradation of CIP and TC by rSLAC

In an enzyme catalysed reaction, pH plays a significant role in the transformation of contaminants (Sun *et al*., 2017). At the different pH levels tested, TC degradation was not greatly affected by pH. However, in the case of CIP, removal efficiency was found to be pH dependent. At pH 4, the degradation rate was low (20%). However, with an increase in pH (5 to 8), the removal percentage improved to 95%. From **Fig. 5.5a**, it is clear that the degradation rate for both antibiotics improved with an increase in the pH of the reaction medium and a maximum degradation level of 95 and 100% was achieved for 5 mg/L of either of the antibiotics (CIP and TC), respectively at pH 8. This is in contrast with other reports where optimal pH for degradation of antibiotics was found to be from low to neutral range (i.e. pH range of 4-7) (De Cazes *et al*., 2014; Suda *et al*., 2012; Sun *et al*., 2017; Yang *et al*., 2017). The result seems promising as the reported pH of urban wastewater and hospital effluents is in the alkaline range (Cruz-Morató *et al*., 2014, 2013).

An investigation of the rSLAC-mediated degradation of CIP and TC revealed that rSLAC alone could degrade 16% and 74% of 5 mg/L of CIP and 5 mg/L TC, respectively. The difference in the degradation efficiency of these antibiotics could be due to the difference in their structure, where TC has a phenol structure which is not present in CIP **(Fig. 5.6)**. However, in order to enhance the oxidative potential of rSLAC, a mediator was added to the reaction. Upon laccase oxidation, mediators form radicals that act as an electron shuttle between the substrate and

laccase thereby expanding the substrate range of the laccase (Cañas and Camarero, 2010). However, in order to develop a sustainable approach of remediation, we used a natural mediator, acetosyringone (AS) in the degradation of the antibiotics. Furthermore, we optimised the concentration of AS (0-300 mg/L) for the degradation of 5 mg/L of CIP and 50 mg/L of TC and achieved ≥95% degradation of CIP and TC at a 200 mg/L concentration of AS **(Fig. 5.5b)**. In another study, quite a high concentration of artificial mediatior, 1-hydroxybenzotriazole (400 mg/L) was used to achieve complete degradation of TC (Sun *et al*., 2017). Results obtained here suggest that rSLAC coupled with AS may be capable of remediating CIP and TC from contaminated environments. This is important since fluroquinolones and tetracyclines are persistent micropollutants. The concentration of these drugs usually varies from ng to µg/L in environment, however, in some cases, effluents released from hospitals or pharmaceutical industries may contain levels of up to mg/L (Becker *et al*., 2016). The presence of antibiotics in aquatic environments stimulate the occurrence of antibiotic resistance genes, which causes severe epidemiological consequences world-wide (Sprenge and Fukuda, 2016; Sun *et al*., 2017; Zhang and Zhang, 2011).

Fig. 5.5c shows that the transformation of CIP decreases from 98% to 60% as the initial CIP concentration increases from 2.5 to 10 mg/L. However, in the case of TC, transformation decreased from 100% to 95% when the concentration was increased from 50 to 200 mg/L. It is worth noting that only 0.4 U rSLAC was used to degrade ≥95% of 5 mg/L of CIP and 200 mg/L of TC, indicating a high catalytic efficiency of rSLAC as also supported by our earlier report (Yadav *et al*., 2018). Comparatively, high dosage, 5 U of fungal laccase were used to achieve complete degradation of 50 mg/L of TC (Sun *et al*., 2017).

Time course for degradation of both antibiotics showed that maximum degradation (i.e. ≥95%) was achieved in 6 h (**Fig. 5.5d**). Remarkably, degradation of 50% of CIP and 85% of TC was achieved within 2 h.

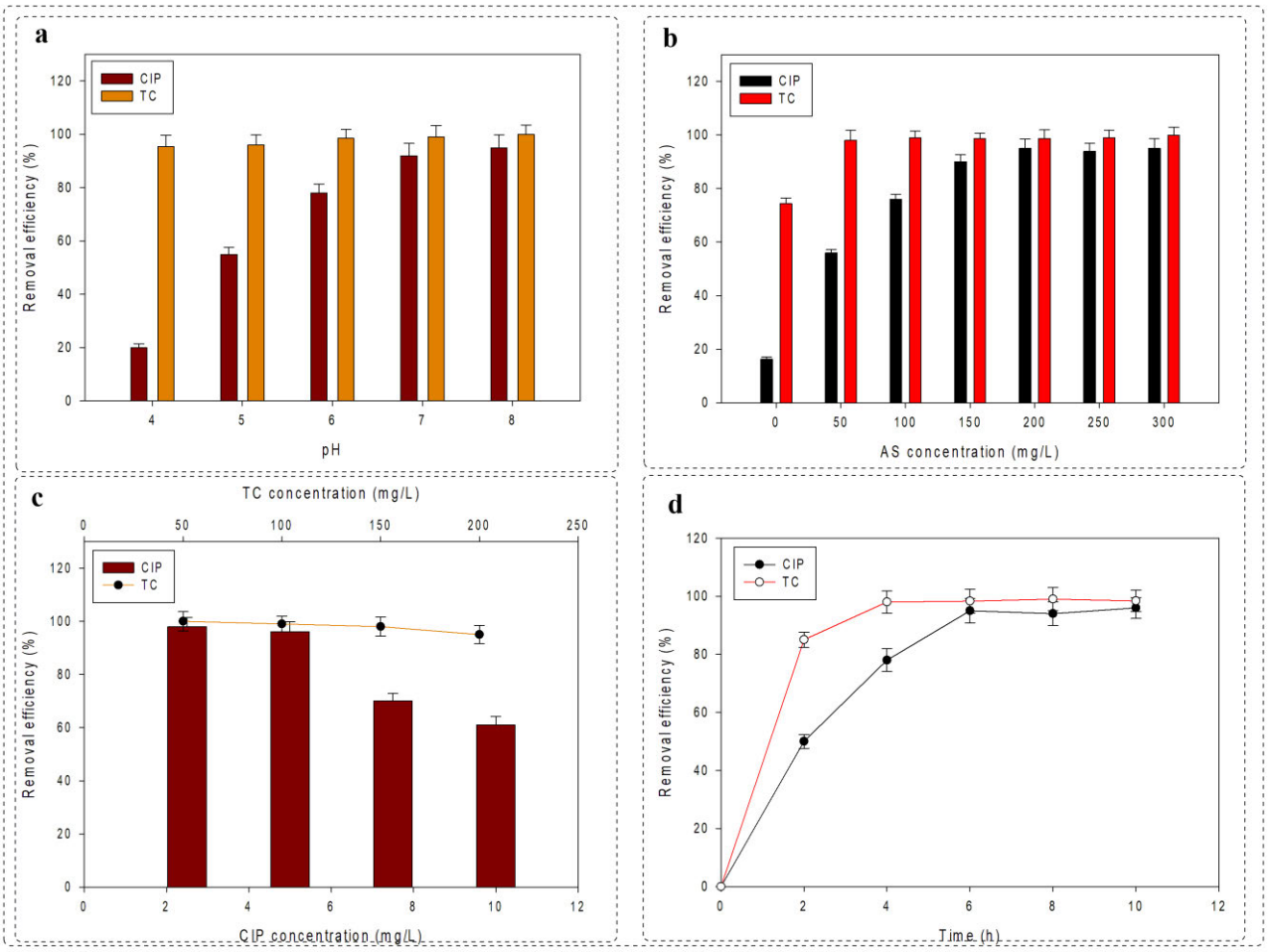


Fig. 5.5 Transformation of ciprofloxacin (CIP) and tetracycline (TC) by rSLAC AS-mediated reaction under varying conditions (a-c) for a 12 h incubation. (a) [CIP]=5 mg/L, [TC]=50 mg/L; [AS]= 200 mg/L, [rSLAC]=0.4 U/mL, pH 4.0-8.0; (b) [CIP]=5 mg/L, [TC]=50 mg/L; [rSLAC]=0.4 U/mL; [AS]=0-300 mg/L, [pH]=8.0; (c) [CIP]=2.5-10 mg/L, [TC]=50-250 mg/L; [rSLAC]=0.4 U/mL, [AS]=200 mg/L, [pH]=8.0; (d) Time course degradation of the antibiotics: [CIP]=5 mg/L, [TC]=100 mg/L, [AS]=200 mg/L, [rSLAC]=0.4 U mL^{-1}, [pH]=8.0. Standard deviations are depicted by error bars and are based on experiments performed in triplicate.

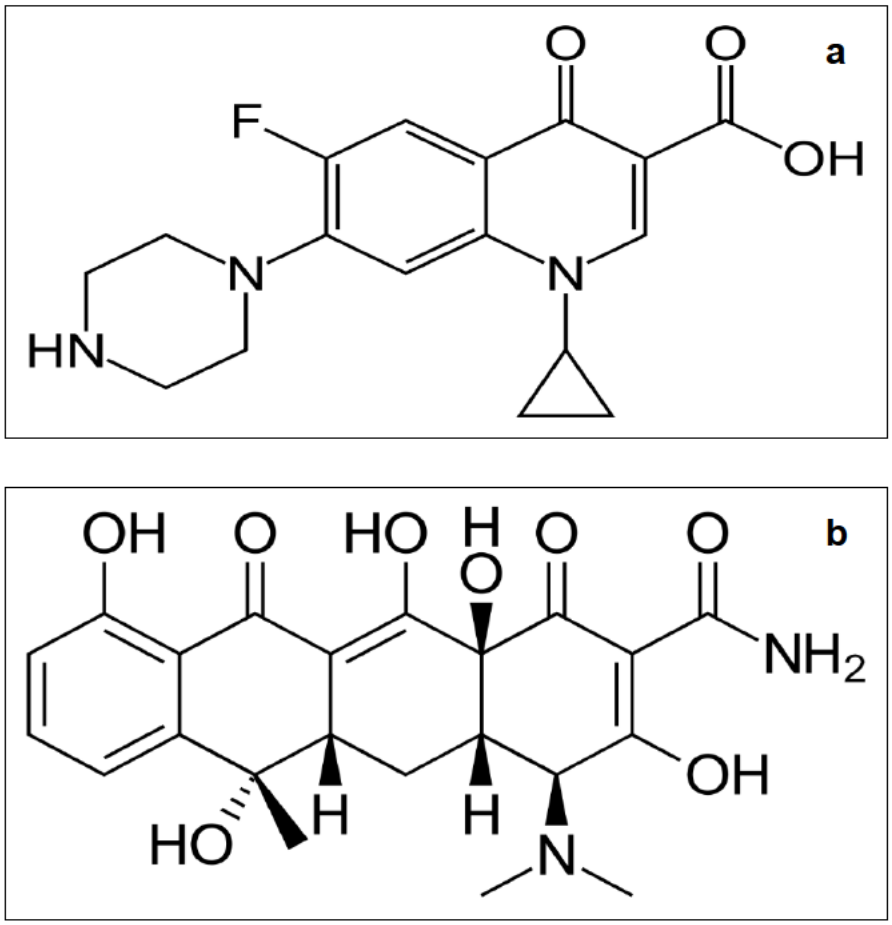


Fig. 5.6 Structure of antibiotics: (a) ciprofloxacin, (b) tetracycline

5.3.4.2 HPLC analysis of degradation products

Fig. 5.7 shows the chromatograms of untreated (reaction without rSLAC) and treated (reaction with rSLAC) CIP and TC solutions. CIP, TC and AS were detected at retention times (t_R) of 5.0, 6.2 and 28.8 min, respectively. However, after rSLAC treatment, the peak of CIP was significantly reduced indicating a low residual concentration of CIP after treatment with laccase. A number of transformation products were observed indicating degradation of CIP. On the other hand, TC treated samples showed complete depletion of TC (t_R=6.2), along with the appearance of at least five transformation products.

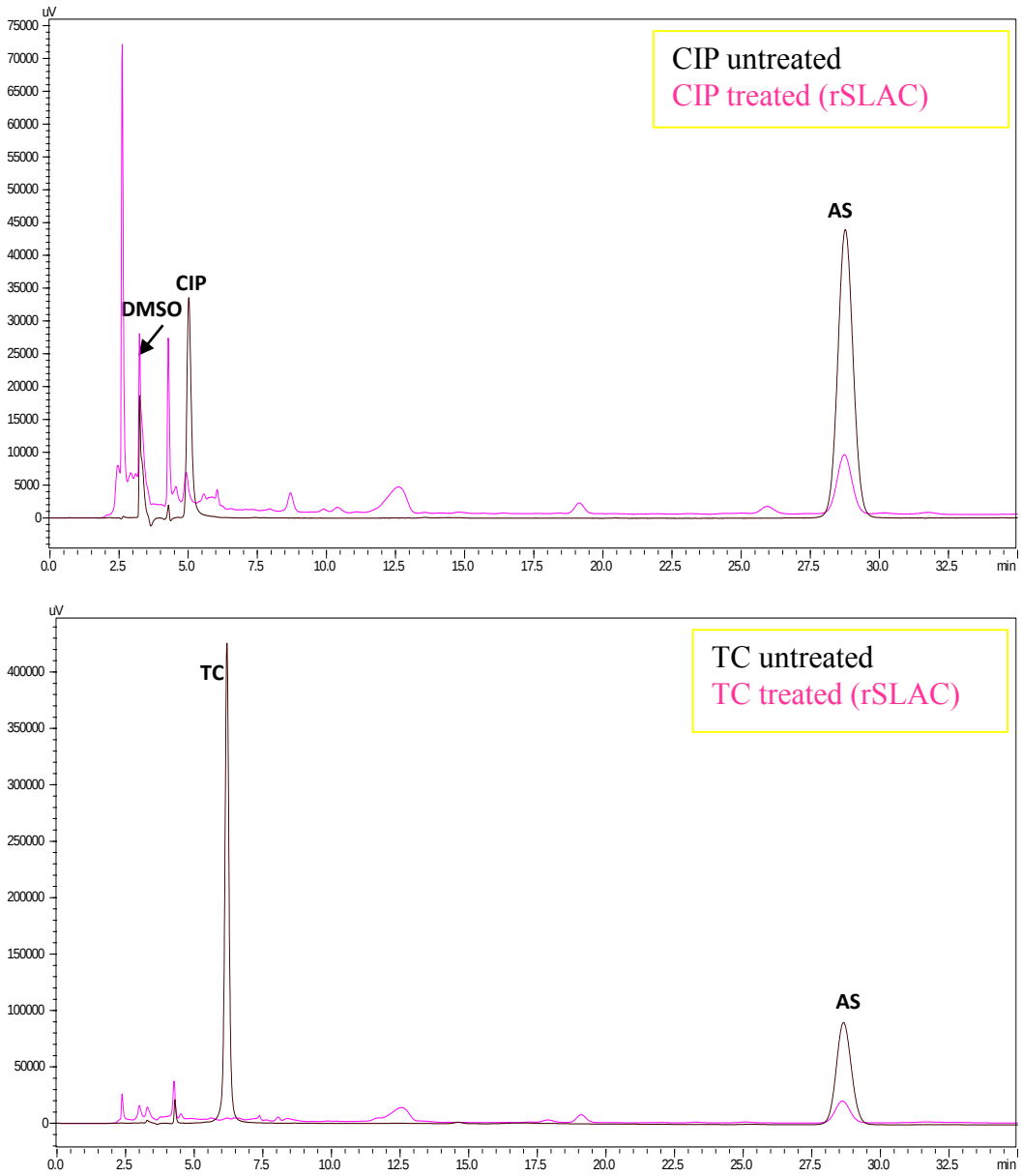


Fig. 5.7 HPLC chromatograms of ciprofloxacin (CIP) (5 mg/L) and tetracycline (TC) (100 mg/L) and their transformation products after rSLAC treatment. The presence of the mediator, acetosyringone (AS), is also indicated on the chromatograms.

5.3.4.3 Residual antibacterial activity of the untreated and treated antibiotics

Assessment of the antibacterial activity of the transformation products generated after treatment is vital in order to evaluate the efficacy of the treatment method. According to several

previous reports, degradation does not always lead to complete mineralisation, resulting in residual antibacterial activity of the degraded parent compounds (De Bel *et al*., 2009; Kusari *et al*., 2009; Marco-Urrea *et al.*, 2009; Pan *et al.*, 2018; Sturini *et al.*, 2012). Therefore, we assessed the antibacterial activity of the untreated and rSLAC treated solutions of CIP and TC by a quantitative (growth inhibition) and qualitative (well diffusion) analysis (**Fig. 5.8a** & **b**). It is evident that the transformation products of CIP and TC was accompanied by considerable reduction in the antibacterial activity (48% reduction in the case of CIP and 99% reduction in the case of TC) as compared to their untreated parent compounds **(Fig. 5.8a)**. In rSLAC treated samples of CIP, a lower growth inhibition was observed which could be due to the residual CIP in the reaction mixture or due to the formation of toxic transformation product(s). Conversely, growth inhibition was completely removed in case of treated TC samples indicating complete removal of antibacterial potency of TC after rSLAC treatment.

In well diffusion assays **(Fig. 5.8b)**, the diameter of the halo around wells reflects the antibacterial potential of the compound(s). **Fig. 5.8b** shows that antibiotic controls of CIP and TC formed distinct zones of inhibition against both Gram-positive *S. aureus* and Gram-negative *E. coli*. Solvents, DMSO (in case of CIP) and distilled water (in case of TC), did not affect the growth of the reference microorganisms (**Fig. 5.8b**). However, in the rSLAC treated CIP samples, the diameter of the zone of inhibition was reduced from 26 mm to 12 mm in the case of *E.coli* and from 16 mm to 12 mm in the case of *S.aureus*. However, no inhibition zone was observed for the rSLAC treated TC samples, indicating complete reduction of antibacterial potency of TC via rSLAC treatment in 6 h. Therefore, it was evident from both the assays that rSLAC degraded the antibiotics and reduced their antibacterial activities. In addition, the results obtained from the qualitative well diffusion assays were consistent with those from the quantitative percentage growth inhibition studies. Though our results report growth inhibition

with *E. coli* and *S. aureus*, a similar trend can also be expected with other bacteria as well, since the mechanism of action of CIP and TC remains the same for all susceptible bacteria (Paul *et al*., 2010).

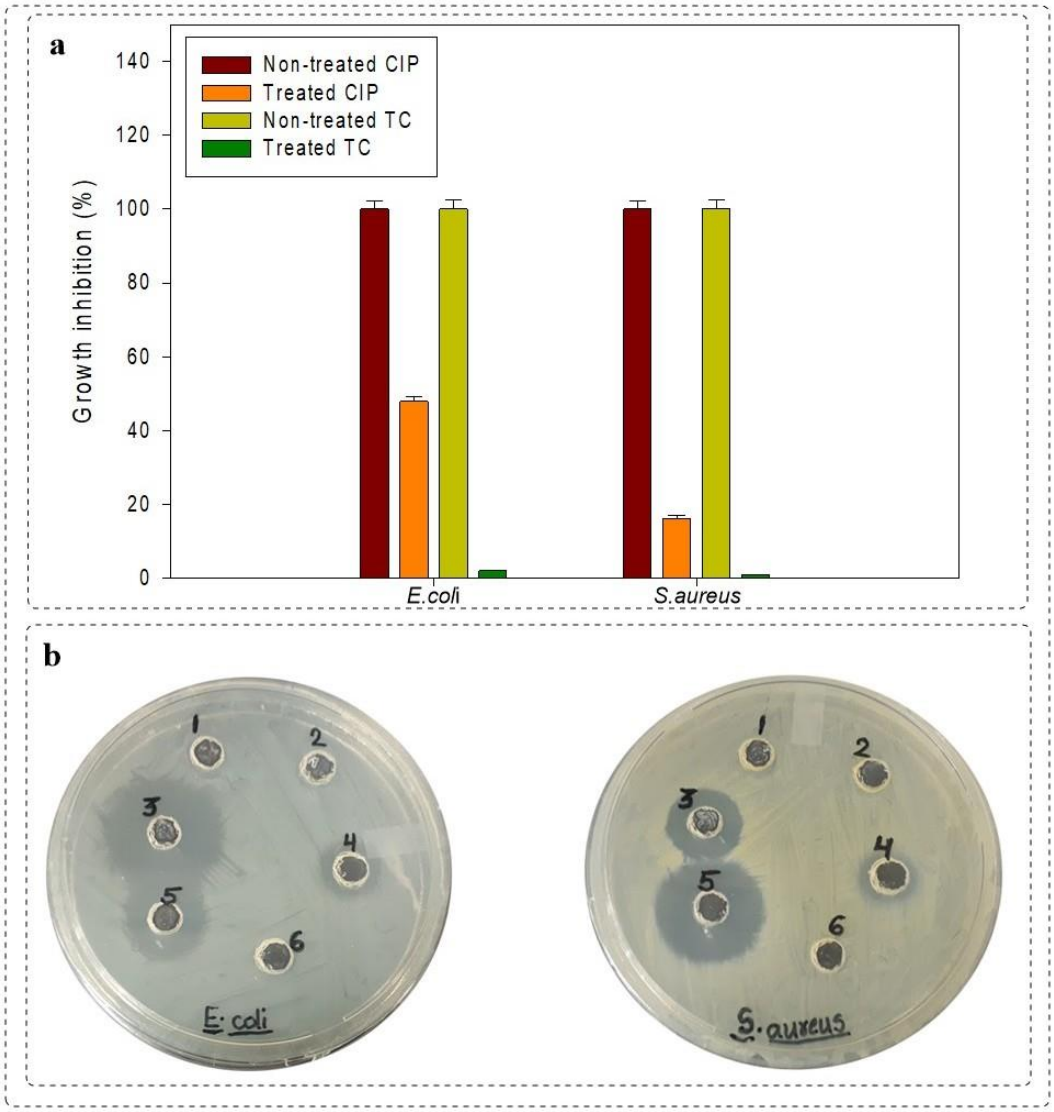


Fig. 5.8 Antibacterial activity of the products of ciprofloxacin (CIP) and tetracycline (TC) treatment using *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 as reference organisms. **(a)** Growth inhibition percentage in relation to untreated antibiotics, **(b)** well diffusion assays.

Solvent controls: **1** DMSO & **2** MilliQ; Non-treated antibiotics: **3** CIP (2.5 mg/L) and **5** TC (100 mg/L); Treated antibiotics: **4** CIP & **6** TC.

5.4. Conclusions

Use of a dual promoter system enhanced the production of rSLAC in *P. pastoris* as compared to a single promoter system. The produced rSLAC removed CIP and TC under the optimised experimental conditions. The degradation products of CIP showed reduction of antibacterial activity against *E. coli* and *S. aureus* while complete removal of antibacterial activity was observed in treated TC. Overall, the dual promoter system could be a viable approach for improving the production of the rSLAC. The rSLAC can be a good candidate for the removal of the persistent antibiotics, CIP and TC, from contaminated environments.

CHAPTER 6

Enzymatic treatment of phenolic pollutants by a recombinant small laccase immobilised on magnetic nanoparticles

Abstract

The small laccase is a good candidate for the remediation of phenolic pollutants and related compounds mainly due to its broad substrate specificity, activity over a broad pH range and its robust nature. However, inability to reuse the free enzyme is a major limitation. In this study, we synthesized magnetic nanoparticles (MNPs), functionalised them by silanization and used them for the covalent immobilisation of a recombinant small laccase (rSLAC) from *Streptomyces coelicolor*. The immobilised recombinant laccase (MNP-rSLAC) was subsequently used for the treatment of phenol, 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP). The enzyme completely removed 80 µg/mL of the selected phenolic compounds within 2 h in the presence of a natural mediator, acetosyringone. The MNP-rSLAC retained >73% residual activity (2,6-dimethoxyphenol as substrate) after 10 catalytic cycles and could be easily recovered from the reaction mixture by the application of magnetic field. Growth inhibition studies using *Escherichia coli* showed that rSLAC-mediated treatment of phenolic compounds reduced the toxicity of phenol, 4-CP and 4-FP by 90, 60 and 55%, respectively. Interestingly, the presence of selected metal ions (Co^{2+}, Cu^{2+}, Mn^{2+}) greatly enhanced the catalytic activity of rSLAC and MNP-rSLAC. This study indicates that immobilised small laccase (MNP-rSLAC) has potential for treating wastewater contaminated with phenolic compounds.

6.1 Introduction

The extensive use of phenolic compounds mainly as herbicides, flame retardants, pesticides, biocides and wood preservatives (Dai *et al*., 2015) has led to their detection in surface water. Removal of these compounds from the contaminated water is necessary due to their low biodegradability (Pera-Titus *et al*., 2004) and toxicity (Liu *et al*., 2012) as well as the pressure to comply with stringent standards against their disposal (Karam and Nicell, 1997). Laccases have been extensively investigated as potential candidates for the remediation of phenolic pollutants. Laccases are multi-copper oxidases which have been gaining a lot of attention mainly due to their ability to catalyse the oxidation of a broad range of substrates without the aid of any cofactors (Upadhyay *et al*., 2016). They use molecular oxygen as an electron acceptor during substrate oxidation, leading to the formation of reactive radicals. The radicals may polymerise and precipitate out of solution which facilitates removal of the pollutant. Alternatively, they can cleave covalent bonds such as C-C, C-O, sometimes assisted by oxidised mediators, leading to the breakdown of pollutants (Huber *et al*., 2018; Kudanga *et al*., 2011). In this sense, laccases, due to their relative promiscuity, can be applied in the bioremediation of several pollutants (Le *et al*., 2016), including phenolic pollutants. However, there are a number of limitations associated with their use as free enzymes which include large consumption, difficulty in separation from the reaction mixture and low recycling capability (Fernandes *et al*., 2017; Ranjan *et al*., 2018). Furthermore, low stability and potential contamination of the free enzyme in wastewater (Rotkova *et al.*, 2009) restricts the industrial use of free laccase in wastewater treatment plants (WWTPs).

Enzyme immobilisation offers an alternative for resolving the challenges associated with free enzymes (Chen, Zou and Hong, 2015), one which could reduce operational expenses (Fortes *et al*., 2017). Enzyme immobilisation can be achieved by physical adsorption or covalent

bonding (Mohamad *et al.*, 2015). Adsorption is a relatively simple method, however, leaching of the enzyme from the immobilised matrix after a few cycles limits its application (Sheldon and Woodley, 2018). In contrast, covalent binding provides an advantage over the adsorption method by stabilising strong bonding between enzyme and the matrix via a chemical reaction, thereby minimising enzyme leaching (Faridi *et al.*, 2017). Among the several immobilisation matrices that have been studied, magnetic nanoparticles (MNPs) have proven to be a promising candidate for the immobilisation of enzymes (Ranjan *et al*., 2018). They possess unique properties such as superparamagnetism, low toxicity, large surface area-to-volume ratio, biocompatibility, and ease of separation by the application of an external magnetic field (Fortes *et al.*, 2017; Mohamed *et al.*, 2017; Pereira *et al.*, 2017; Ranjan *et al*., 2018). However, formation of agglomerates due to magnetic dipole interactions (Villa *et al*., 2016) and the unstable nature (in acidic medium) of naked iron magnetic particles, could greatly affect their longevity and reusability.

Functionalisation via chemicals and biological materials could enhance and optimise the surface properties of MNPs such as dispersibility, biocompatibility and biodegradability (Xu *et al*., 2014). In addition, surface functionalisation provides the anchoring surface which mediates the attachment of functional groups, leading to an engineered surface with selective affinity towards diverse bio-compounds (Dehnavi *et al.*, 2015). In one recent study, polydopamine (PDA) coating was used to functionalise Fe_3O_4 nanoparticles for immobilisation of a fungal laccase (Zhang *et al*., 2017). However, the high cost of dopamine could potentially impact its usage in industrial catalysis (Wang *et al*., 2016). Silanization is a viable alternative owing to its responsivity, high stability, low cytotoxicity as well as the ease of surface chemical transformation. In addition, no specialized equipment is required, making silanization an ideal

method for functionalisation (Faridi *et al*., 2017). Despite these favourable properties, silanization has not been used in the immobilisation of laccase.

Therefore, in this study silanization was explored for surface functionalisation of MNPs followed by immobilisation of rSLAC onto the functionalised MNPs. The immobilised rSLAC was then applied in the treatment of selected phenolic pollutants.

6.2 Materials and methods

6.2.1 Materials

Phenol, 4-chlorophenol (4-CP), 4-fluorophenol (4-FP), 4-aminoantipyrine (4-AAP), potassium ferricyanide, 2,6-dimethoxyphenol (2,6-DMP) and acetosyringone (AS) were purchased from Sigma-Aldrich (South Africa). Orthophosphoric acid and acetonitrile were purchased from Merck (South Africa). All chemicals were of analytical grade and were used without any further purification. The rSLAC was produced and purified as previously described (Yadav *et al*., 2018).

6.2.2 Determination of laccase activity and protein concentration

Laccase activity was determined using 2,6-DMP as a substrate, as previously described (Yadav *et al*., 2018). The oxidation of 2,6-DMP (2 mM) was analysed by measuring the increase in absorbance at 477 nm (ε = 14,800 M^{-1} cm^{-1}) in Tris-HCl buffer (20 mM, pH 8.0). One unit of laccase activity was defined as the amount of enzyme required for the oxidation of 1 µmol of substrate per minute. Protein concentration was determined by the Lowry method (Lowry *et al*., 1951) using bovine serum albumin as a standard.

6.2.3 Synthesis and silanization of MNPs

MNPs were synthesized by a chemical co-precipitation method as previously described (Ranjan *et al*., 2018). Synthesized MNPs were collected by the application of magnetic field and washed with ethanol followed by Milli-Q water. Finally, MNPs were dried under vacuum overnight at 60 °C. Surface modification of the dried MNPs was carried out using (3-aminopropyl)-triethoxysilane (APTES) as described by Faridi *et al.* (2017). One gram of MNPs were dispersed in a mixture of 20% (w/v) APTES and 4 mL glycerol, and the resulting mixture was heated at 90 °C for 2 h under nitrogen sparging and stirring. The mixture was then cooled, sonicated for 10 min and the modified MNPs collected using a magnet followed by washing with ethanol and Milli-Q water and dried overnight under vacuum at 60 °C.

6.2.4 Immobilisation of rSLAC on modified MNPs

The carboxyl groups of rSLAC were treated with 3-(3 dimethylaminopropyl) N′-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) as described by Faridi *et al.* (2017). Briefly, 3 mg of EDC was added to a 5 mL solution of rSLAC and the mixture was incubated at room temperature (22±3°C) for 1 h with gentle shaking followed by the addition of 3 mg of NHS. Thereafter, 10 mg of MNPs were added to the mixture and the incubation was continued for further 3 h. The binding efficiency of rSLAC onto silanized MNPs was determined by the difference in concentration of the free enzyme after immobilisation against the initial enzyme concentration (Faridi *et al*., 2017).

6.2.5 Characterisation of MNPs

The presence of functional groups on the surface of MNPs was assessed by FT-IR spectroscopy (Perkin Elmer, USA). The morphology of MNPs was studied by field emission scanning electron microscopy (FE-SEM) using a Zeiss Gemini instrument (Germany).

6.2.6 Biochemical and kinetic characterisation of free and immobilised rSLAC

The optimum pH for activity of free and immobilised rSLAC was determined by conducting enzyme assays in the pH range 5.0-9.0, in 1.0 pH value increments [20 mM sodium acetate buffer (pH 5.0), 20 mM Tris-HCl buffer (pH 6.0-8.0) and 20 mM glycine-NaOH buffer (pH 9.0)] at 80 °C. Optimum temperature for free and immobilised rSLAC was determined by performing enzyme assays at various temperatures (40 to 100 °C) at pH 8.0. The enzyme activity was presented in terms of relative activity, with the highest catalytic activity set at 100%.

Kinetic parameters (K_m and k_{cat}) were determined using 2,6-DMP (50-2000 µM) as a substrate for the immobilised rSLAC and compared with the free rSLAC (Yadav *et al*., 2018). The reactions were carried out in Tris-HCl buffer (20 mM, pH 8.0) and oxidation of 2,6-DMP was determined at 477 nm (ε= 14,800 M^{-1} cm^{-1}). The experiments were performed in triplicate.

6.2.7 Effect of metal ions and organic solvents on catalytic activity of free and immobilised rSLAC

The effect of different metal ions (Li^{+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Mo^{6+}, Al^{3+}, Mg^{2+}) on catalytic activity of free and immobilised rSLAC was evaluated. Initially, enzymes were pre-incubated in presence of 10 and 30 mM of individual metal ions for 30 min (Sondhi *et al*., 2014) and thereafter enzyme activity was measured using 2,6-DMP as substrate as previously described. The enzyme activities of free and immobilised rSLAC without metal ions were considered as controls (100% activity). Similarly, the enzyme (free or immobilised) was pre-incubated for 30 min in organic solvents (20 and 50% of methanol, ethanol, acetone, dioxane and DMSO) and the residual activity determined using 2,6 DMP as substrate. All experiments were performed in triplicate.

6.2.8 Storage stability and reusability of the immobilised rSLAC

The storage stability of the free and immobilised rSLAC was evaluated by storing the enzyme in Tris-HCl buffer (20 mM, pH 8) at 4 °C for 30 days. Sampling was done after every 6 d and the enzyme activity was determined using 2,6-DMP as a substrate. Enzyme activity at time zero was taken as 100% relative activity.

The reusability of the immobilised rSLAC was evaluated by recovering the MNP-rSLAC with a magnet after each cycle and washing three times with Tris-HCl buffer (20 mM, pH 8.0) to prepare for the subsequent cycle. Enzyme activity was determined using 2,6-DMP as the substrate. The enzyme activity for the first cycle was considered as 100% and relative activities of the subsequent cycles were calculated with respect to the activity of the first cycle.

6.2.9 Treatment of phenol, 4-CP and 4-FP by free and immobilised rSLAC

The rSLAC (2 U) was added to a reaction mixture (1 mL total volume) consisting of Tris-HCl buffer (50 mM, pH 8.0), phenols or halogenated phenols (80 µg/mL each), and AS (8 µg/mL) as mediator, and the mixture was incubated at 37 °C in the dark with gentle shaking (50 rpm). A negative control was also set in parallel where a denatured enzyme (in case of free rSLAC) and bare MNPs (in case of immobilised rSLAC), were used and incubated under identical conditions. Samples were collected every 5 d (until day 30) to analyse the residual concentrations of phenolic compounds by colorimetric and HPLC analysis. Furthermore, the effects of pH (4.0-8.0), mediator concentrations (2-10 µg/mL) and reaction time, on the degradation of phenolic compounds (80 µg/mL each), were studied.

6.2.9.1 Analysis of the residual phenolic compounds by a colorimetric method

Residual phenolic compound concentrations were measured using a simple colorimetric assay as described by Modaressi *et al.* (2005). The assay is based on the electrophilic attack of the

phenolic compound by a primary amine (4-AAP) under alkaline conditions, and its subsequent oxidation by potassium ferricyanide to a red quinone-type dye (Faust, 1967; Al- Kassim *et al.*, 1994). The reaction mixture consisted of 700 µL of Tris-HCl buffer (100 mM, pH 8), 300 µL laccase treated phenolic compounds, 10 µL 4-AAP (0.1 M) and 10 µL potassium ferricyanide solution (0.2 M) and was incubated at 25 °C for 15 min, with gentle shaking (50 rpm). The extent of removal of the compounds was determined spectrophotometrically as the relative decrease of the maximum absorbance at 510 nm. Percentage removal of the phenolic compounds was calculated according to Singh *et al.* (2017) as follows:

$$\text{Removal efficiency (\%)} = (A_i - A_f)/A_i \times 100\%$$

where, A_i is the initial absorbance of the phenolic compounds and A_f is the final absorbance of the phenolic compounds (after rSLAC treatment).

6.2.9.2 Analysis of the residual phenolic compounds by HPLC

HPLC analysis was performed using a Shimadzu HPLC system from Dionex (Massachusetts, USA) equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Chromatographic separation was achieved on C18 column (Sunfire, Waters, Ireland) using 0.1% orthophosphoric acid (A) and acetonitrile (B) as mobile phase, flow rate 1.0 mL/min, injection volume of 10 µL and an oven temperature of 40 °C. The gradient was set up as follows: 2% B to 100% B (0-20 min), 100% B to 2% B (20-22 min), 2% B (22-30 min). The peaks for phenolic compounds as well as the transformation products were observed at 254 nm.

6.2.10 Toxicity analysis

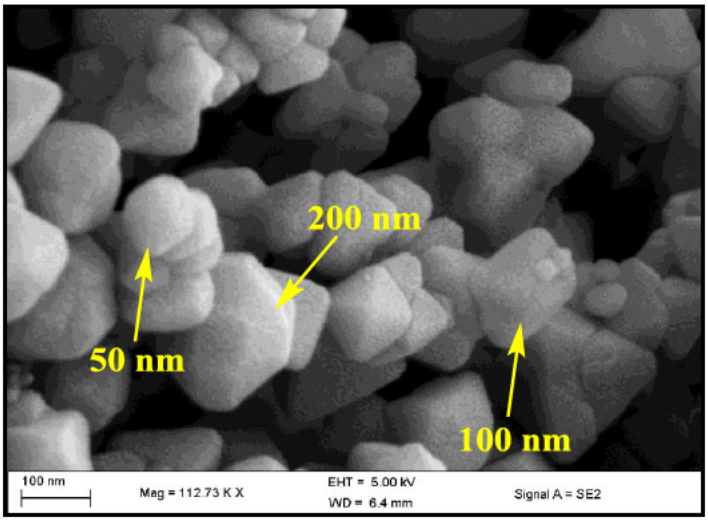
Reduction in toxicity of phenolic compounds after rSLAC treatment was monitored by bacterial growth inhibition studies as described by Murugesan *et al.* (2010) using *Escherichia coli* ATCC 25922 as test organism. Individual phenolic compounds (80 µg, total volume of 900 µL) and their degradation products (total volume of 900 µL) were incubated separately in sterile nutrient broth (9 mL) with 100 µL of 0.1 OD_{600} overnight culture of *E. coli* ATCC 25922. The test tubes were incubated with shaking (200 rpm) in the dark at 37 °C and bacterial growth was monitored spectrophotometrically at 600 nm. Results were presented as percentage growth inhibition relative to the control sample (containing 9 mL nutrient broth 100 µL of 0.1 OD_{600} overnight culture of *E. coli* ATCC 25922 and 900 µL saline).

6.3 Results and discussion

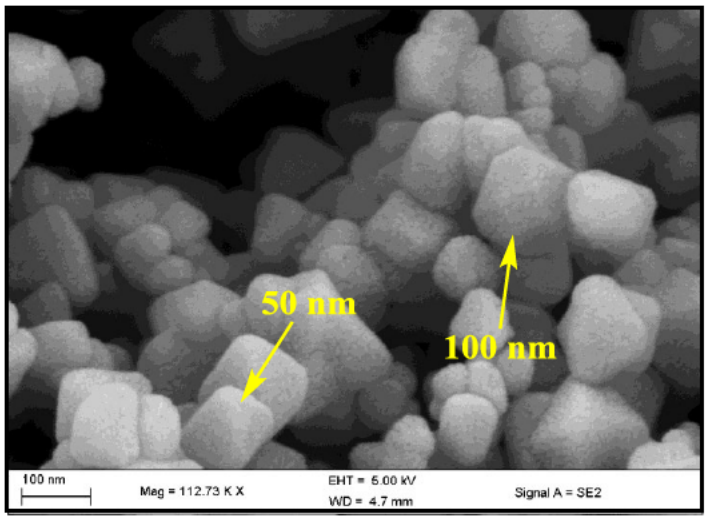
6.3.1 Characterisation of MNPs and immobilised MNPs

FE-SEM images of bare and silanized MNPs showed no evidence of morphological changes after silanization. Further, FE-SEM images of MNPs also revealed that their diameter was in the range of 50-200 nm (**Fig. 6.1**). FT-IR spectra confirmed the presence of characteristic peaks for MNP, MNP/APTES and MNP/APTES/rSLAC (**Fig. 6.2**). A high intensity peak at 589 cm^{-1} was observed in uncoated nanoparticles, which is characteristic peak for MNPs (Zhang *et al*., 2007). Interestingly, we also observed that the intensity of MNP peaks (589 cm^{-1}) decreased after subsequent grafting of APTES and APTES/rSLAC, confirming the successful grafting of APTES and rSLAC onto the MNPs (**Fig. 6.2**). The appearance of a peak in the region of 2360 cm^{-1} is due to Si-H silane (**Fig.6.2b,c**), which further confirmed the grafting of APTES onto MNPs. The appearance of a peak at 1648 cm^{-1} in MNP/APTES/rSLAC (**Fig. 6.2c**) could be due to the $CO\text{-}NH_2$ group, which confirmed the formation of bond between enzyme and Si-

MNP (Faridi *et al*., 2017). The appearance of a broad peak obtained at 3200-3500 cm^{-1} in all the three spectra depicts the presence of hydroxyl groups on the surface of nanoparticles (Ma *et al*., 2003). Furthermore, we also noticed that the peak intensity at 3200-3500 cm^{-1} has subsequently increased after grafting of APTES and APTES/rSLAC onto MNPs, which could be due to the physical and chemical adsorption of water molecules after each coating. Thus, peaks shown by FT-IR confirmed the successful synthesis of MNPs and grafting of APTES and rSLAC onto the surface of nanoparticles.



a



b

Fig. 6.1 FE-SEM images showing surface morphology of **(a)** bare and **(b)** functionalised MNPs

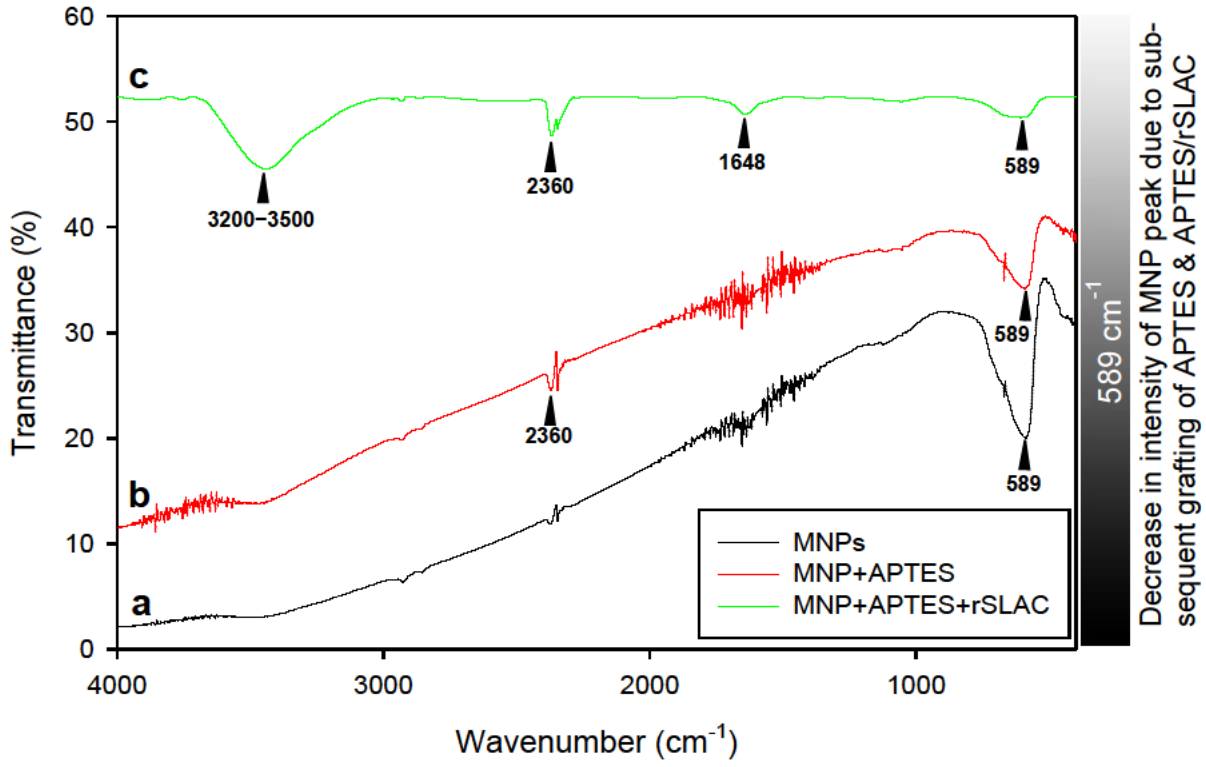


Fig. 6.2 FTIR spectra of MNPs, MNP+APTES and MNP+APTES+rSLAC.

6.3.2 Effect of pH and temperature on the catalytic activity of free and immobilised rSLAC

The catalytic activity of free and immobilised rSLAC was evaluated in the pH range 5.0 - 9.0 and maximum activity was observed at pH 8.0 for both free and immobilised enzyme (**Fig. 6.3a**). However, the immobilised enzyme showed higher catalytic activity at pH 5.0 and 6.0 compared to the free enzyme. The enhanced catalytic activity over a broad pH range could be attributed to the MNP support. Similar results have been observed by Zhang *et al.* (2017), who also reported that the immobilized enzyme showed broader pH-activity profile. The optimum temperature for both enzymes was 80 °C (**Fig. 6.3b**). It was also observed that the immobilised rSLAC retained higher catalytic activity (~20%) at 100 °C compared to the free enzyme, which suggests enhanced thermostability after immobilisation. Elevated temperature could change the conformation and enzyme-substrate interaction of free enzymes, causing a negative effect

on catalytic performance. On the other hand, immobilisation can provide protective effect against increase in temperature by reducing conformational flexibility of enzymes. Therefore, enhanced thermostability of rSLAC immobilised on modified MNP could be attributed to the enzyme rigidity owing to the anchorage between the support and protein, promoted by covalent bond formation (Costa *et al*., 2019).

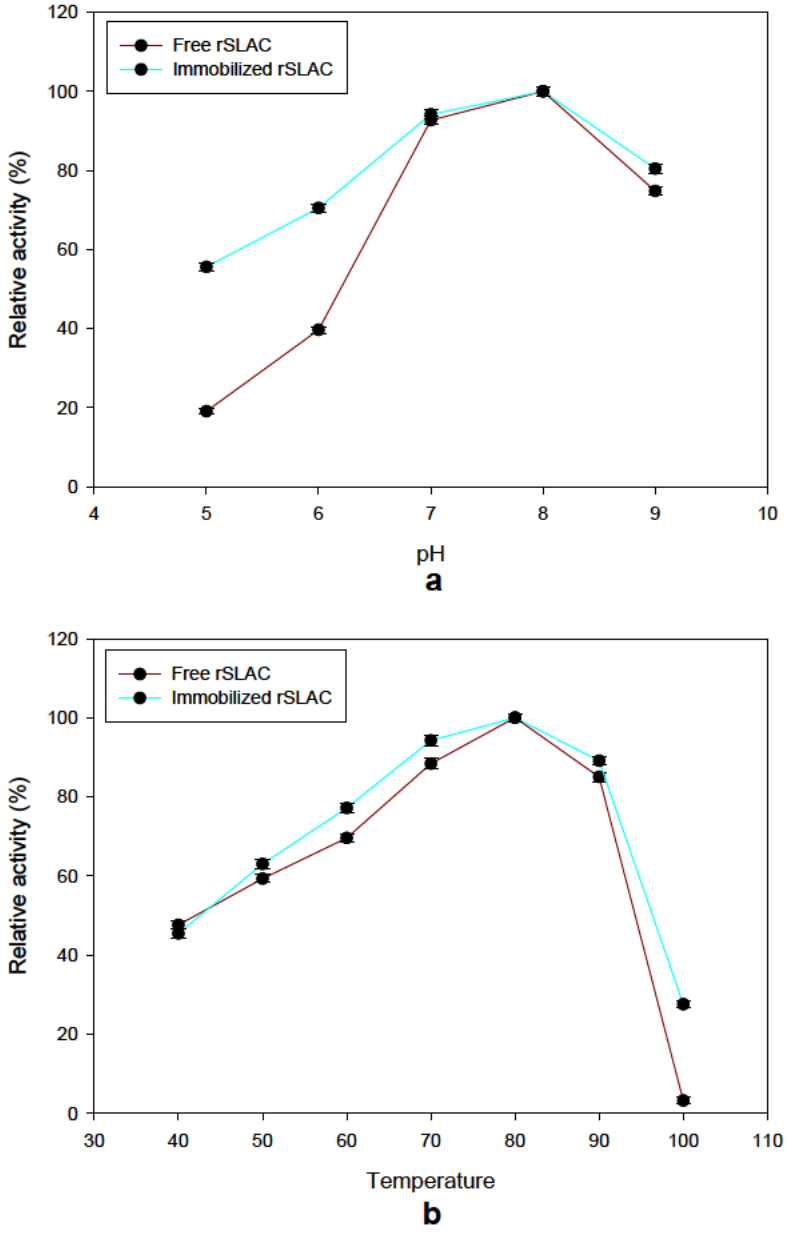


Fig. 6.3 Effect of **(a)** pH and **(b)** temperature on the catalytic activity of free and immobilised rSLAC using 2,6 DMP as a substrate. Values are means ± standard deviation (n=3).

6.3.3 Determination of kinetic parameters for free and immobilised rSLAC

The Michaelis constant (K_m) for the free and immobilised enzymes were 54.05 and 54.17 µM, respectively while the maximum velocities (V_{max}) were 25.88 and 26.26 µM mg^{-1} min^{-1}, respectively. The slight increase in K_m value for the immobilised rSLAC could be due to the diffusional limitation, which resulted in a lower enzyme-substrate affinity. An increase in K_m values after immobilisation of laccase has also been reported in several studies (Wang *et al*., 2010, 2013). Comparable V_{max} values of free and immobilised rSLAC suggest that the immobilisation process did not compromise catalytic activity.

6.3.4 Effect of metal ions and organic solvents on free and immobilised rSLAC

Heavy metal ions are common environmental pollutants and are often found in industrial wastewater (Zeng *et al*., 2017). These metal ions could activate or inhibit the catalytic activity of enzymes (Rodríguez Couto *et al*., 2005), which can affect the enzymatic remediation process. Therefore, the effects of different metal ions on the catalytic activity of free and immobilised rSLAC were evaluated. Interestingly, it was observed that Co^{2+}, Cu^{2+} and Mn^{2+} ions greatly stimulated the catalytic activity of free and immobilised rSLAC (**Table 6.1**), where the order of enhancement in rSLAC activity was Co^{2+} > Mn^{2+}> Cu^{2+}. Activation of rSLAC by Cu^{2+} is not surprising as copper is an essential component of the active site (Chakroun *et al.*, 2010; Lu *et al.*, 2012). The results are consistent with those obtained using a recombinant polyphenol oxidase, where Co^{2+} stimulated a higher catalytic efficiency compared to Cu^{2+} (Moon *et al*., 2018). In addition, apart from copper, manganese also has a known role in inducing the transcription and activity of laccases (Baldrian *et al*., 2000). However, the other metal ions only slightly improved the activity of the enzymes, with Al^{3+} reducing activity at 30 mM **(Table 6.1**). Enhanced catalytic activity of laccase by several metal ions *viz.*, Mn^{2+}, Mg^{2+}, Mo^{6+} , Ca^{2+} has also been reported in a previous study (Zhao et al., 2012). The negative effect

of Al^{3+} observed here is consistent with a previous report where the suppressive effect of this trivalent metal ion towards laccase-Cu^{2+} inhibited the degradation of 4-nitrophenol (Lu *et al.*, 2012). Overall, our results suggest that the presence of some metal ions in wastewater could facilitate the remediation of phenolic compounds.

Table 6.1 Effect of different metal ions on catalytic activity of free and immobilised rSLAC. All values are means ± SD (n=3).

Metal ions	**Concentrations (mM)**	**Relative activity (%)**	
		Free rSLAC	**MNP-rSLAC**
Control	-	100 ± 1.16	100 ± 1.62
Li^{+}	10	104 ± 0.58	103 ± 1.53
	30	114 ± 2.08	112 ± 1.17
Co^{2+}	10	563 ± 2.00	556 ± 2.52
	30	604 ± 2.08	598 ± 1.54
Cu^{2+}	10	179 ± 2.07	175 ± 2.06
	30	253 ± 2.53	250 ± 3.04
Mn^{2+}	10	315 ± 2.04	311 ± 3.52
	30	333 ± 2.53	329 ± 1.50
Ca^{2+}	10	120 ± 3.08	116 ± 2.40
	30	141 ± 2.02	138 ± 2.50
Mo^{6+}	10	126 ± 2.48	128 ± 1.28
	30	137 ± 2.10	136 ± 1.56
Al^{3+}	10	117 ± 0.82	119 ± 2.08
	30	94 ± 1.57	96 ± 1.58
Mg^{2+}	10	114 ± 2.59	112 ± 1.58
	30	119 ± 1.55	121 ± 1.18

Organic solvents have been known to inhibit enzyme activity via protein unfolding (Afreen *et al*., 2017). Results on the effect of different organic solvents showed that catalytic activities of free and immobilised rSLAC were not affected by the solvents tested (at 20 and 50%) except

for DMSO **(Table 6.2)**. The activities of both the free and immobilised enzymes were significantly increased in the presence of dioxane (**Table 6.2**). To the best of our knowledge, stimulatory effect of dioxane has not been reported, which could be a novel feature of rSLAC. The rSLAC retained full activity at 20 and 50% methanol, ethanol and acetone whilst a minor effect was observed with DMSO (2% inhibition at 20% DMSO and 8% inhibition at 50% DMSO). In a previous study with rCotA laccase, however, DMSO significantly inhibited the enzyme (62% inhibition with 10% DMSO) (Wang *et al.*, 2015) which indicates that the rSLAC has a much better tolerance to DMSO. As some of the pollutants, such as the phenolics studied here, polycyclic aromatic hydrocarbons (PAHs), organophosphorus pesticides and azo dyes, are only soluble in organic solvents, the use of immobilised rSLAC could be useful for their detoxification.

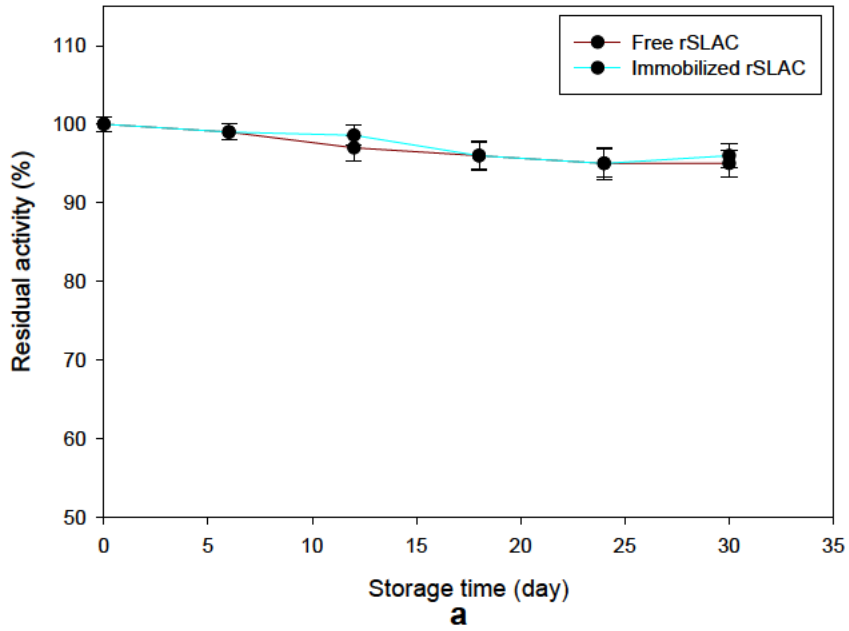
Table 6.2 Effect of different organic solvents on catalytic activity of free and immobilised rSLAC. All values are means ± SD (n=3).

Organic solvent	**Concentration (%, v/v)**	**Relative activity**	
		Free rSLAC	MNP-rSLAC
Control	-	100 ± 1.16	100 ± 1.62
Methanol	20	110 ± 3.08	109 ± 1.56
	50	104 ± 1.58	106 ±1.54
Ethanol	20	115 ± 2.06	113 ± 1.57
	50	109 ± 2.56	110 ± 3.62
Acetone	20	109 ± 2.54	107 ± 2.07
	50	101 ± 2.06	103 ± 2.53
Dioxane	20	123 ± 1.74	122 ± 2.48
	50	198 ± 3.05	194 ± 2.58
DMSO	20	98 ± 2.00	99 ± 3.06
	50	92 ± 1.18	98 ± 1.16

6.3.5 Storage stability and recycling efficiency of immobilised rSLAC

The use of enzymes in an industrial application is greatly affected by the cost of their production, thus, enzymes with long-term storage stability and high recycling efficiency are preferred (Alex *et al*., 2014). To this end, storage stability and reusability of immobilised rSLAC were assessed. The free and immobilised enzymes were stored in Tris-HCl buffer (20 mM, pH 8) at 4 °C and their activities monitored every 6 days over a period of 30 days. Both enzymes retained ≥95% of initial activity (**Fig. 6.4a**).

The recycling efficiency of immobilised rSLAC was evaluated in a batch operation mode for 10 cycles, using 2,6-DMP as a substrate. The MNP-rSLAC retained >73% of its initial activity after 10 consecutive cycles (**Fig. 6.4b**). The decrease in laccase activity after repeated cycles could be due to the leaching of enzyme from the immobilised support or accumulation of reaction products that may reduce mass transfer efficiency and make the enzyme less accessible by its products. A fungal laccase immobilised on MNPs functionalised by PDA retained 70% of its initial activity after 10 catalytic cycles (Zhang *et al*., 2017). This implies that salinization, with favourable properties outlined earlier, could be a viable alternative functionalisation approach. The remarkable storage stability along with the reusability of the MNP-rSLAC during 10 successive cycles, emphasises its potential for large-scale application.



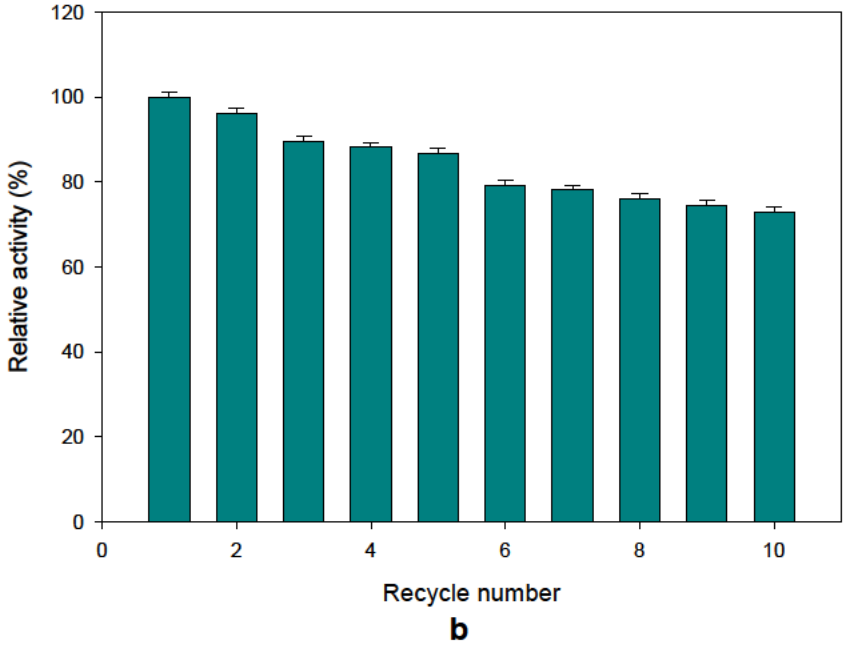


Fig. 6.4 (a) Storage stability of free and immobilised rSLAC at 4°C **(b)** Reusability of immobilised rSLAC (MNP-rSLAC). Values are means ± standard deviation (n=3).

6.3.6 Removal of phenol, 4-CP and 4-FP by rSLAC

In order to enhance transformation, the effect of various parameters, such as pH, mediator concentrations and time, on the removal of the phenolic compounds, was investigated.

6.3.6.1. Effect of pH on removal of phenolic compounds

pH is one of the critical factors for any enzyme-catalysed reaction. Therefore, the effect of pH on rSLAC-mediated removal of phenol, 4-CP and 4-FP was evaluated in the pH range of 4.0-8.0. Interestingly, it was observed that ≥98% removal was achieved over a wide pH range of pH 6.0-8.0 (**Fig. 6.5**). However, the removal of phenolic compounds was relatively low at acidic pH; it increased substantially in the alkaline range (**Fig. 6.5**). Since the pH of wastewater effluents are in the range of 5.5-9.8 (Gaitan *et al*., 2011; Nair *et al*., 2013), the application of rSLAC in real wastewater conditions could be feasible.

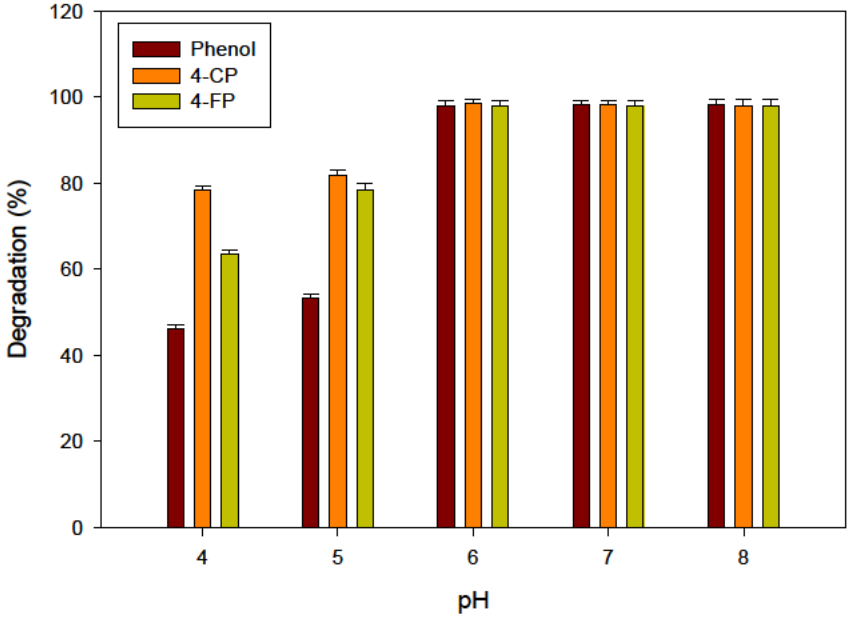


Fig. 6.5 Effect of pH on the degradation of phenol, 4-CP and 4-FP (80 mg/L) with 2 U/mL rSLAC at 37 °C. Values are means ± standard deviation (n=3).

6.3.6.2. Effect of mediator concentration on removal of phenolic compounds

The inclusion of mediators has been the general approach in enhancing laccase-mediated transformation of recalcitrant compounds (Murugesan *et al.*, 2010). Therefore, the effect of different mediators such as acetosyringone (natural) and 1-hydroxybenzotrialzole (synthetic), on the removal of phenolic compounds was evaluated. Both mediators showed similar effects (data not shown), therefore further experiments were carried out with the natural mediator due to its eco-friendly properties (Kunamneni *et al.*, 2008). It was observed that complete removal was achieved at 8 mg/L concentration of acetosyringone (**Fig. 6.6**). A previous study used a much higher concentration of artificial mediator (ABTS; 642.5 mg/L) to achieve the complete degradation of 4-CP (64.28 mg/L) (Chakroun *et al.*, 2010).

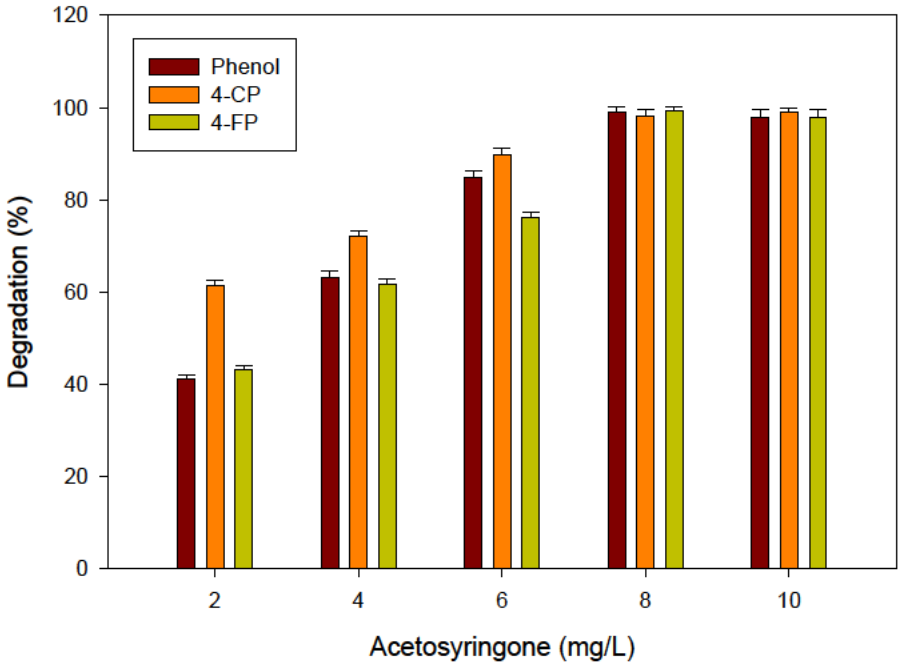


Fig. 6.6 Effect of mediator (acetosyringone) concentration on the degradation of phenol, 4-CP and 4-FP (80 mg/L) using rSLAC. Values are means ± standard deviation (n=3). The reaction was performed at 37 °C, pH 8 for 2 h.

6.3.6.3. Effect of time on removal of phenolic compounds

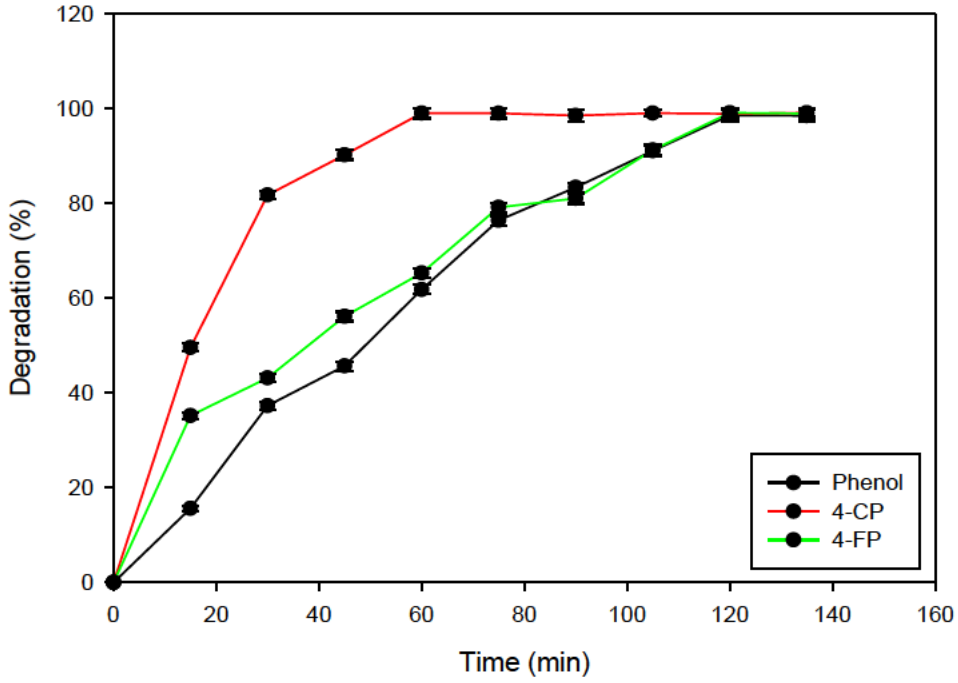


Fig. 6.7 Time dependent degradation profile of phenol, 4-CP and 4-FP using rSLAC in the presence of a redox mediator, acetosyringone. Reaction conditions: 80 mg/L phenol, 4-CP or 4-FP, 2U rSLAC, 8µg/mL acetosyringone, 37 °C, pH 8. The residual concentrations of phenol, 4-CP and 4-FP were determined colorimetrically (510 nm). Data presented as the mean ± SD (n=3).

The transformation of phenol, 4-CP and 4-FP were performed using the purified rSLAC (2 U or 40 µg/mL) at optimal pH and mediator concentration. Remarkably, ≥98% removal of all the phenolic compounds was achieved within 2h (**Fig. 6.7**). Moreover, removal of 61, 98 and 65% was observed for phenol, 4-CP and 4-FP, respectively, in only 1 h of reaction time. However, in a previous study, 100% degradation of 2-chlorophenol was achieved in 4 h using 10 U of laccase (Gaitan *et al.*, 2011). Similarly, Zhang *et al.* (2017) achieved 86% degradation of 4-CP in 2h using 250 mg/L of immobilised laccase. The requirement of the low amount of laccase for the complete removal of phenolic

compounds, in this study, could be attributed to the high catalytic efficiency of the rSLAC, as previously reported (Yadav *et al*., 2018). In addition, we also noticed that the removal of halogenated phenols was attained in less time compared to the non-halogenated phenol (**Fig. 6.7**). This is consistent with previous reports where 4-chlorophenol was degraded much faster than phenol (Arica *et al*., 2009; Liu *et al*., 2012).

6.3.7 HPLC analysis of transformation products

The transformation of phenol, 4-CP and 4-FP by rSLAC was further analysed by HPLC. The retention time for phenol was t_R=8.7 min; the peak completely disappeared after laccase-catalysed transformation and several transformation products were formed which eluted at longer retention times **(Fig. 6.8c)**. The retention times for the elution of 4-CP and 4-FP were observed at t_R=10.6 and t_R=9.6 min, respectively in control samples **(Fig. 6.9a** and **6.10a)**. Similarly, the peaks for 4-CP and 4-FP disappeared in test samples and multiple peaks appeared after delayed retention times **(Fig. 6.9c** and **6.10c)**. The delayed retention times observed in all test samples could be due to the formation of oligomeric products (Cabana *et al*., 2007). Similar findings were observed in laccase-mediated transformation of triclosan (Murugesan *et al*., 2010).

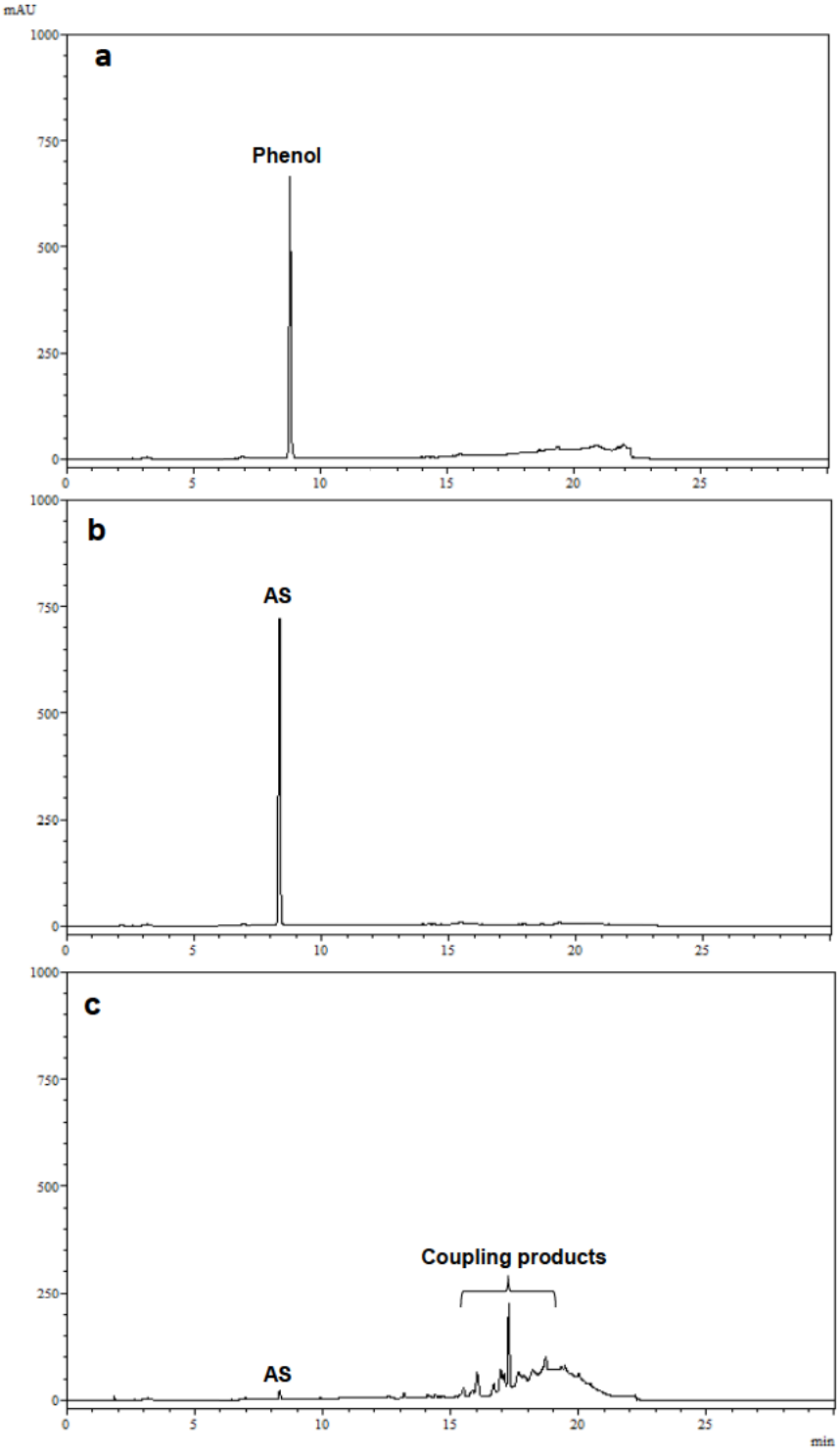


Fig. 6.8 HPLC chromatograms of **(a)** Phenol, **(b)** acetosyringone (AS) and **(c)** rSLAC-AS treated phenol. Experimental conditions: [Phenol, 4-CP and 4-FP] =700 mg/L, [rSLAC =18 U], [Acetosyringone] =70 mg/L, at 37 °C, pH 8.

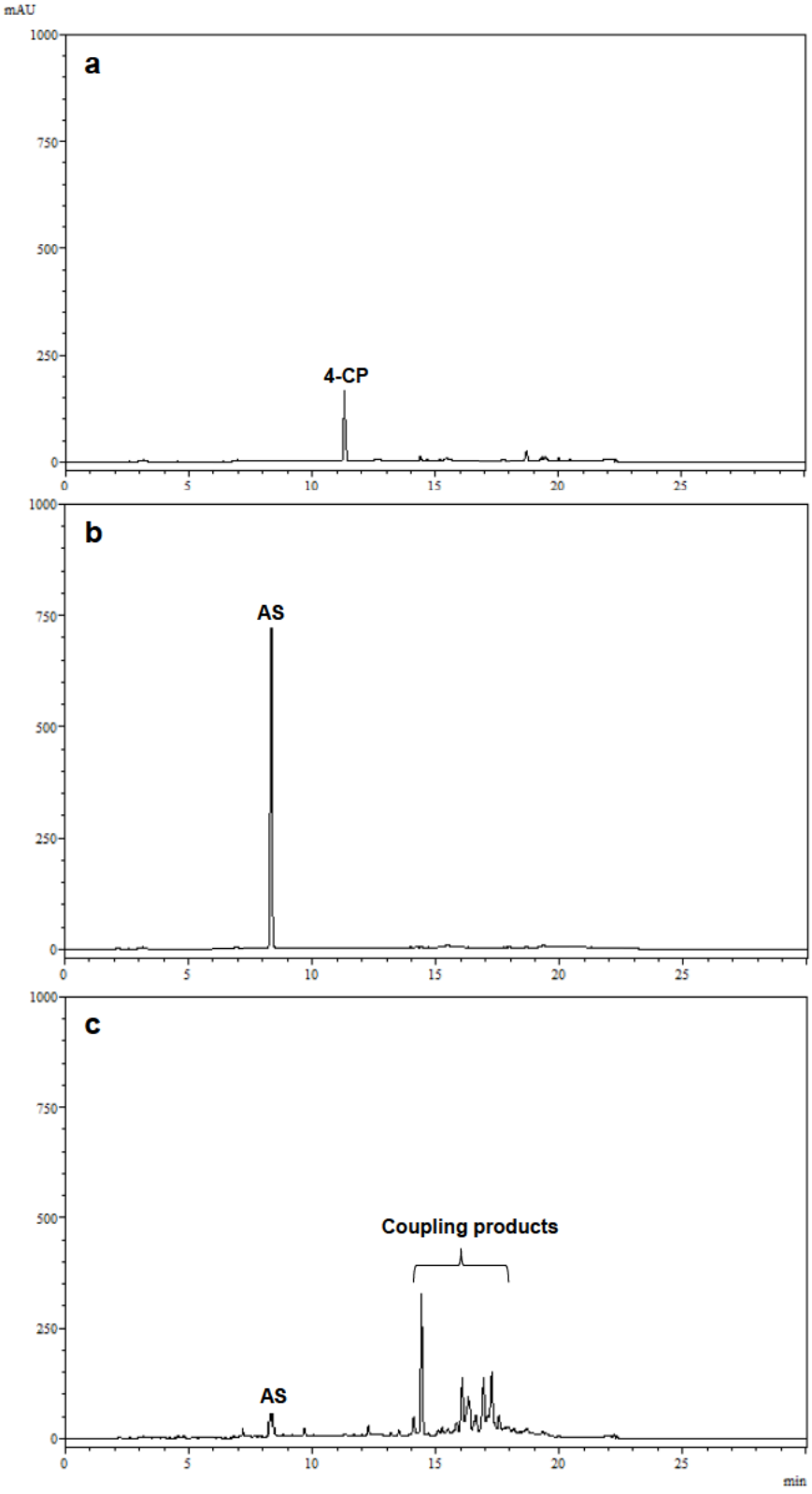


Fig. 6.9 HPLC chromatograms of **(a)** 4-CP, **(b)** acetosyringone (AS) and **(c)** rSLAC-AS treated 4-CP. Experimental conditions: [Phenol, 4-CP and 4-FP] =700 mg/L, [rSLAC =18 U], [Acetosyringone] =70 mg/L, at 37 °C, pH 8.

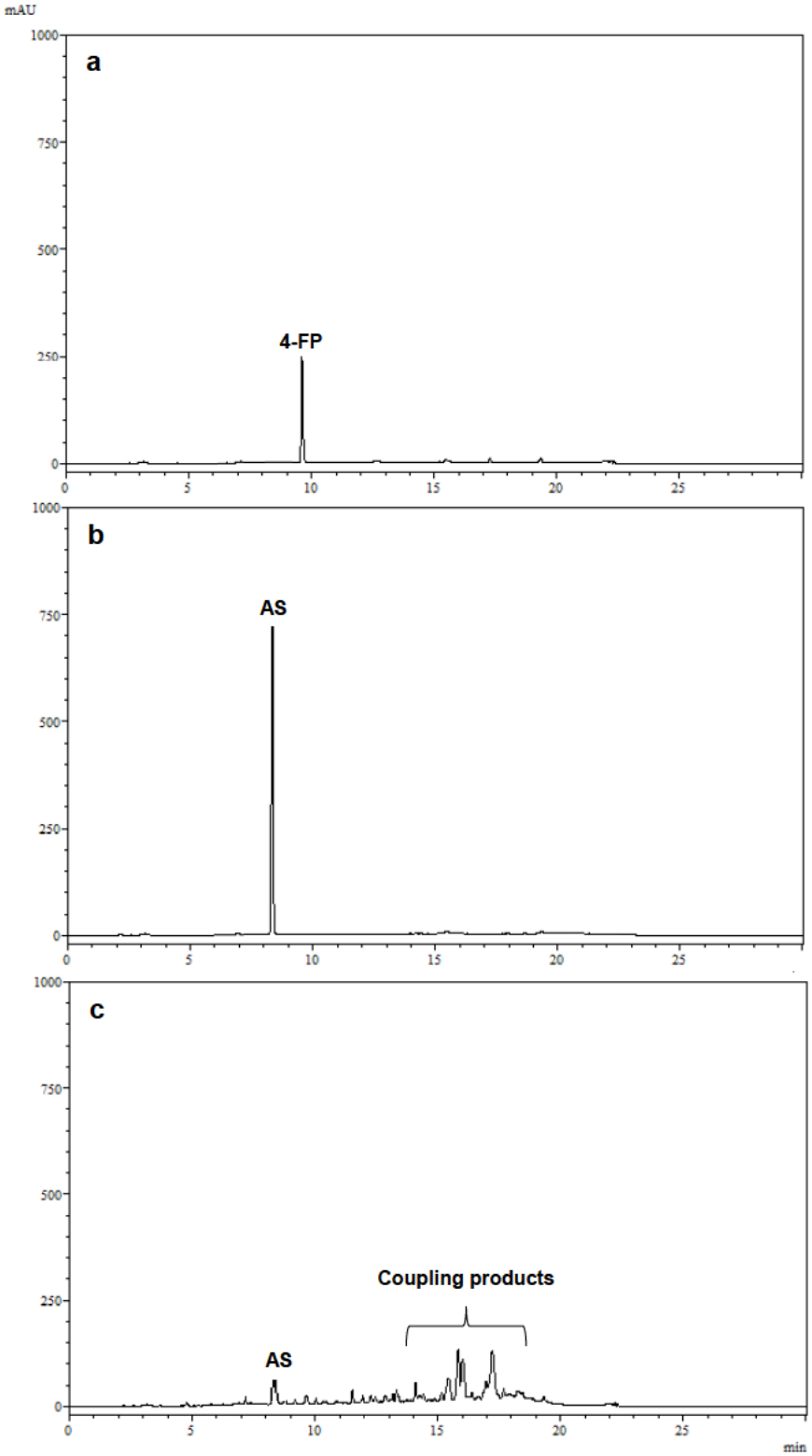


Fig. 6.10 HPLC chromatograms of **(a)** 4-FP, **(b)** acetosyringone (AS) and **(c)** rSLAC-AS treated 4-FP. Experimental conditions: [Phenol, 4-CP and 4-FP] =700 mg/L, [rSLAC =18 U], [Acetosyringone] =70 mg/L, at 37 °C, pH 8.

6.3.8 Toxicity analysis

Toxicity analysis of phenolic compounds and their products were assessed by performing the bacterial growth inhibition studies using *E. coli* ATCC 25922 as test organism. It is well known that phenolic compounds increase bacterial cell membrane permeability which leads to the release of their cell wall components and ultimately cell death (Denyer, 1995; Mcdonnell and Russell, 1999). In the present study, 80 µg/mL of the phenolic compounds used (phenol, 4-CP and 4-FP) completely inhibited the growth of *E. coli* ATCC 25922 (**Fig. 6.11**). However, the transformation products inhibited the bacterial growth by 10, 40 and 45% for phenol, 4-CP and 4-FP, respectively. This suggests that the toxicity of phenol, 4-CP and 4-FP transformation products were reduced by 90, 60 and 55%, respectively. The halogenated compounds *viz.*, 4-CP and 4-FP showed higher toxicity compared to the phenol. Higher toxicity of halogenated phenolic compounds was also observed in a previous study, where it was reported that an increase in the number of chlorine atoms was associated with an increase in bacterial growth inhibition (Wang *et al*., 2008). On the other hand, it was observed in a previous study that degradation products were more toxic than the parental compounds (Lu *et al*., 2015). In this study we observed that after the rSLAC treatment of phenolic compounds the products were precipitated out of the reaction mixture, which could be due to the polymerisation of the products. This could provide an additional advantage for the physical separation of the polymerized products from a wastewater sample using filtration methods. Separation of precipitates by filtration methods has also been previously reported (Ba *et al*., 2014).

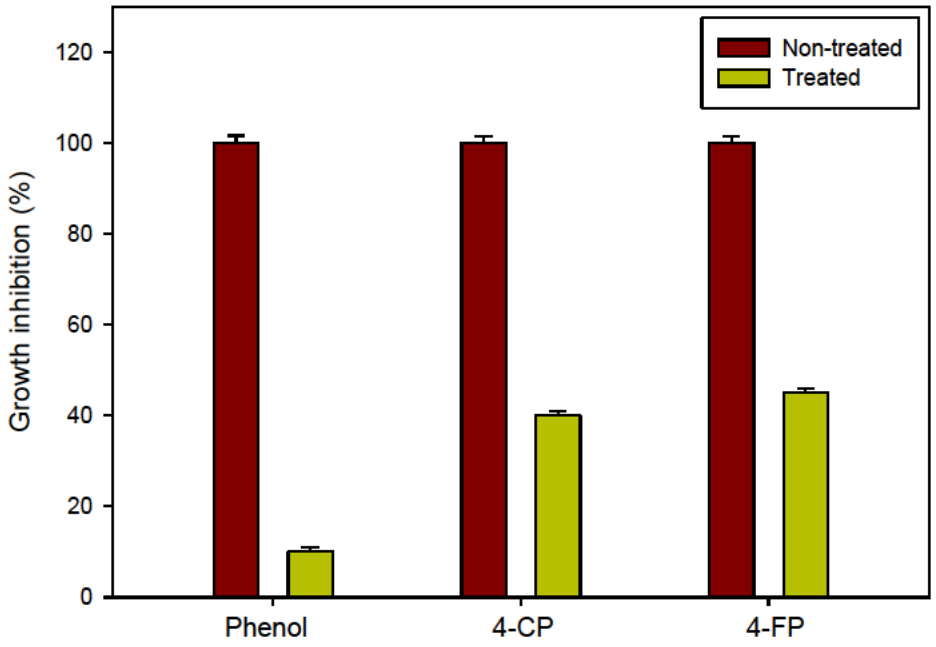


Fig. 6.11 Effect of non-treated and rSLAC treated phenolic compounds on bacterial growth inhibition. Data is presented as mean ± SD from triplicate experiments.

6.4. Conclusions

In this study rSLAC was for the first time successfully immobilised onto MNPs functionalised by silanization. In the presence of a natural mediator, acetosyringone, ≥98% transformation of phenol and halogenated phenols was achieved within 2 h. The enzyme showed great potential for the removal of phenolic compounds within a wide pH range, significantly reducing toxicity of the compounds. These findings suggest that immobilised rSLAC could be a candidate for the sustainable bioremediation of phenolic compounds in wastewater.

CHAPTER 7

Laccase-catalysed surface functionalisation of chitosan and coconut fibres for the production of active biomaterials

Abstract

Multiple functionalities can be imparted onto biomaterials using various synthetic and biological approaches. In this study, two biological materials (chitosan and coconut fibres) were engineered with an antimicrobial functionality using the small laccase (SLAC) from *Streptomyces coelicolor* A3(2). The recombinant SLAC (rSLAC) oxidised functional molecules to corresponding radicals which reacted with the lignin moieties and amino groups of the coconut fibres and chitosan, respectively. rSLAC-mediated modification resulted in the appearance of broad absorption band around 380 and 450 nm of the UV/Vis spectra of grafted chitosan films, which could be attributed to reaction between o-quinone and amino groups of chitosan. FTIR spectrum of grafted biomaterials showed new aromatic skeletal vibrations as well as phenolic absorption bands indicating conjugation of allelochemicals onto chitosan and coconut fibres. Antibacterial activities of grafted biomaterials were found to be superior than their ungrafted counterparts. Growth inhibition of 55-60% against *Escherichia coli* and *Staphylococcus aureus* was observed when catechol was grafted onto chitosan or coconut fibres. However, comparatively lower growth inhibition (5-40%) was observed for pyrogallol-functionalised materials. Overall, the results suggest that the rSLAC can be exploited for the functionalisation of coconut fibres and chitosan films to enhance antibacterial properties.

7.1. Introduction

Oxidative enzymes are known for the conversion of phenolic derivatives into quinones. These active radical species can either undergo nucleophilic reaction by reacting with amino groups of chitosan (CS) or can react with one another (oligomerisation). However, two types of reactions with quinones and amines are commonly reported in literature, namely Michael or Schiff base addition or oligomer-forming reactions (Aljawish *et al.*, 2012). Enzymes such as peroxidases and laccases target lignin's phenolic sites and generate a radical-rich surface onto which molecules of interest can be grafted (Nyanhongo *et al.*, 2010). Laccases, unlike peroxidases do not require cofactors (such as H_2O_2), and laccase catalysis is generating a lot of research interest because it requires only oxygen as a co-substrate and water is the only by-product produced (Riva, 2006). Owing to its non-specific nature, laccase-mediated functionalisation is a versatile approach whereby several phenolic compounds can be grafted to impart different functionalities such as antimicrobial, hydrophobicity, improving tensile strength or mechanical properties (Fillat *et al.*, 2012). Antimicrobial activity of hydroxylated phenols such as catechol and pyrogallol is well documented in literature (Jeong *et al.*, 2009; Tinh *et al.*, 2016; Lima *et al.*, 2016; Amato *et al.*, 2018) . These allelochemicals are structurally similar except pyrogallol has an extra -OH group when compared to catechol (which has two -OH groups) (Kocaçalişkan *et al.*, 2006). Grafting of these antimicrobial phenols onto suitable substrates prevents their loss via leaching (Widsten *et al.*, 2010). Biomaterials such as coconut fibres and chitosan are ideal substrates for functionalisation due to the presence of phenolic sites of lignin rich (40-45%) coconut fibres (Thakur *et al*., 2015) and hydroxyl and amine groups of chitosan (Dash *et al*., 2011).

Chitosan is a natural polysaccharide and is a promising biomaterial due to its non-toxicity, good biocompatibility and biodegradability (Božič *et al*., 2012) which can be regarded an

excellent natural material capable of replacing conventional packaging materials. Similarly, coconut fibres are biodegradable materials, which contain lignin moieties that can be easily be oxidised by laccase to form radicals. At the same time suitable functional molecules can be oxidised by laccase to facilitate functionalisation through radical-radical coupling onto the oxidised lignocellulose surface. These enzymatic approaches for modifying materials are now being intensively investigated as alternatives to chemical methods which involve the use of hazardous chemicals and harsh reaction conditions (Witayakran and Ragauskas, 2009). Therefore, unlike enzymatic approaches, chemical processes require proper handling and disposal procedures which tend to increase the cost of the product (Kalia *et al.*, 2013).

In the present study, we adopted a green approach to modify the surface of chitosan and coconut fibres with allelochemicals using a recombinant small laccase (rSLAC) as a catalyst. The functionalised materials were characterized by scanning electron microscopy (SEM), Fourier transform-infrared (FT-IR) spectroscopy and ultraviolet-visible (UV-Vis) spectrophotometry to confirm the grafting. The grafted conjugates were then assessed for their antibacterial activity against selected ATCC bacterial strains.

7.2. Experimental

7.2.1. Materials and reagents

Coconut palm (*Cocos nucifera*) was purchased from a local shop (Durban, South Africa). Catechol, pyrogallol, azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), nutrient broth, nutrient agar, chitosan (degree of acetylation, 85%) were purchased from Sigma-Aldrich, South Africa. All other chemicals used in this study were of analytical grade. The rSLAC from *Streptomyces coelicolor* A3(2), *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were obtained from the Cape Peninsula University of Technology, South Africa,

and are currently deposited in the stock culture collection in the Department of Biotechnology and Food Technology, Durban University of Technology. Recombinant small laccase (rSLAC) was produced and purified as previously described in section **4.2.5** and **4.2.6.**

7.2.2. Determination of laccase activity and protein concentration

Laccase activity was determined as previously described (Yadav *et al.*, 2018) using ABTS (1 mM) as a substrate. The oxidation of ABTS (1mM) in acetate buffer (20 mM, pH 4.0) was analysed by measuring the increase in absorbance at 420 nm (ε = 36,000 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required for the oxidation of 1 µmol of substrate per minute. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

7.2.3. Preparation of chitosan films

Chitosan films were prepared according to Bożič *et al*. (2012). Briefly, a 1% (w/v) chitosan solution was prepared and was centrifuged to remove undissolved materials. The pH of chitosan solution was adjusted to pH 5.8-6.0 using 5 N NaOH. The chitosan was then cast onto 7 cm-diameter glass petri dishes and oven-dried overnight at 45 °C to form a chitosan film. Neutralisation of chitosan films was performed by immersing them in 1 M NaOH for 2 h, which was then followed by washing with water and phosphate buffer (100 mM, pH 6.5), and stored at 4 °C.

7.2.4. Grafting of coconut fibres

Prior to grafting experiments, coconut fibres were extracted from the fruit of coconut palm and were first washed with a mild detergent followed by distilled water to remove water soluble

impurities. Fibres were extracted with acetone in a Soxhlet extractor for 24 h to remove organic impurities.

Biografting reactions were carried out according to Thakur *et al.* (2016) with slight modifications. The reaction mixture consisted of 200 mg of coconut fibres in 50 mM acetate buffer pH 4, 3.5 % (w/v) allelochemical, and 50 U of laccase. The reaction mixture was incubated at 37 °C for 12 h with constant shaking (50 rpm). A control reaction was set up under the same conditions in the absence of the enzyme. Post biografting, the fibres were washed with distilled water and extracted with 50 mL acetone using a Soxhlet extractor for 12 h to remove any unbound phenolics. The fibres were then vacuum-dried to a constant weight.

7.2.5. Functionalisation of chitosan

Homogenous and heterogenous grafting were performed according to Bozîč *et al.* (2012) with slight modifications.

7.2.5.1 Homogenous grafting

The reaction mixture (30 ml total volume) contained chitosan (1% w/*v*), phenolic compound (10 mM), and laccase (50 U), and the pH was adjusted to pH 4.5. The chitosan solution was heated to 30 °C, followed by the addition of the allelochemical with constant stirring. After the homogenous distribution of the mixture, laccase was added slowly with constant stirring and allowed to react for 24 h at 30 °C (under atmospheric conditions). Thereafter, the solution was centrifuged at 5000 x g for 10 min at 4 °C to remove the conjugates. The resultant supernatant was then cast onto petri dishes and dried in an oven at 45 °C. The dried films were peeled off and stored at 4 °C until required for further analysis.

7.2.5.2 Heterogenous grafting

The chitosan films were incubated in 100 ml buffer (100 mM pH 4.5) containing catechol or pyrogallol (10 mM) and laccase (50 U) under constant shaking conditions (50 rpm) at 30 °C. Thereafter, the films were rinsed with ethanol and water to ensure the removal of the physically associated allelochemicals. Control chitosan films were performed without laccase in the reaction mixture.

7.2.6. Structural characterisation of functionalised materials

7.2.6.1. SEM, FTIR and TGA analysis of coconut fibres and chitosan

Surface morphology of coconut fibres and chitosan films, before and after functionalisation, were examined by SEM using a Zeiss Gemini instrument (USA). Functionalised biomaterials were first coated with gold prior to analysis, thereafter the micrographs were taken at 5 kV with magnification of 200 times. Grafting of functional groups onto the surface of functionalised fibres and chitosan films were confirmed by infrared spectra recorded on a Fourier Transform Infra-Red spectrometer (FTIR; Perkin Elmer, USA) with an attenuated total reflection (ATR) attachment. Sixteen scans were recorded in %T mode over a range of 400-4000 cm^{-1} and a resolution of 4 cm^{-1}.

Thermogravimetric analysis (TGA) was performed on a thermal analyser (Perkin Elmer, USA) at a heating rate of 10 ºC/min in an inert atmosphere.

7.2.6.2 UV/Vis spectrophotometry

Enzymatic conjugation of functional molecules (catechol and pyrogallol) onto chitosan was studied using a UV/Vis spectrophotometer (ThermoFischer Scientific, USA) (Fras-Zemljič *et al.*, 2011). The reaction mixture consisted of the functional molecule (10 mM) and chitosan

(1% w/v) in a 50 mM phosphate buffer (pH 6.5) with SLAC (50 U) (test reaction) or without rSLAC (control reaction). Aliquots (200 µl) were taken every 10 minutes till 1 h and reaction between the allelochemical and the chitosan measured by scanning at 300-800, using a microplate reader (ThermoFischer Scientific, USA).

7.2.7. Antibacterial properties of the natural phenols

The antibacterial properties of the allelochemicals (catechol and pyrogallol) were evaluated on two ATCC strains (*Escherichia coli*, *Staphylococcus aureus*) as per Fillat *et al.* (2012). *Staphylococcus aureus* and *E. coli* were selected as representative of Gram-positive and a Gram-negative bacteria, respectively. The bacterial strains were inoculated in 5 ml of LB medium supplemented with different concentrations (0–25 mM) of the catechol or pyrogallol and the cultures were incubated overnight at 37°C, shaking at 200 rpm. Thereafter, the optical density OD_{600} nm was measured as an estimate of the growth of the bacterial strain. Percentage of growth inhibition was calculated using the following formula:

Growth inhibition (%) = 100- (**B**/**A***100)

where, **B** is OD_{600} nm of the culture of a bacterial strain with a allelochemical at a given concentration and **A** is OD_{600} nm of the same culture of a bacterial strain without any added allelochemical.

7.2.8. Antibacterial activity of functionalised derivatives

Antibacterial properties of functionalised chitosan derivatives were evaluated according to ASTEM E2149-01 as described by Bożiĉ *et al.* (2012) with slight modifications. Overnight grown bacterial cultures of *S. aureus* or *E. coli* were added dropwise to the ungrafted and grafted chitosan films or coconut fibres, such that the bacteria were evenly distributed onto the films. Bacteria from the chitosan (CHS) films or coconut fibres were recovered by transferring the films aseptically into a saline solution (0.9% w/v) which was then shaken in an orbital shaker for 1 h. Subsequently, 100 µl was plated onto nutrient agar plates and incubated overnight at 37°C. Efficacy of the functionalised materials was determined by calculating the reduction in viable counts as compared to the initial viable count. Percent reduction in colony forming unit was determined by using the following formula:

Percent reduction rate (**R%**) = **B** – **A**/ **A** * 100

Where, **A**= The number of bacteria from the control or untreated biomaterials, and **B** = the number of bacteria recovered from the functionalised biomaterials

7.3. Results and Discussion

7.3.1. Characterisation of untreated and grafted coconut fibres

7.3.1.1. Scanning electron microscopy (SEM)

Scanning electron micrographs revealed prominent changes in the surface morphology of biografted coconut fibres as compared to their untreated counterparts **(Fig 7.1)**. As evident from **Fig.7.1**, laccase-mediated grafting had transformed the surface of smooth untreated coconut fibre to a rough, irregular and a bulky surface. Grafted coconut fibres had a fractured surface owing to enzymatic grafting of phenolic molecules. The roughness can be attributed to

the polymerisation reactions due to the availability of phenoxy radicals in the reaction mixture. The result was consistent with findings from previous studies performed with coconut fibres (Thakur *et al.*, 2015; Thakur *et al.*, 2016). Transformation in surface morphology is an indication of the attachment of catechol/pyrogallol onto the surface of coconut fibres as a result of laccase treatment. This change in the surface morphology may ultimately lead to a change in the functionality of the untreated coconut fibres (Thakur *et al.*, 2012).

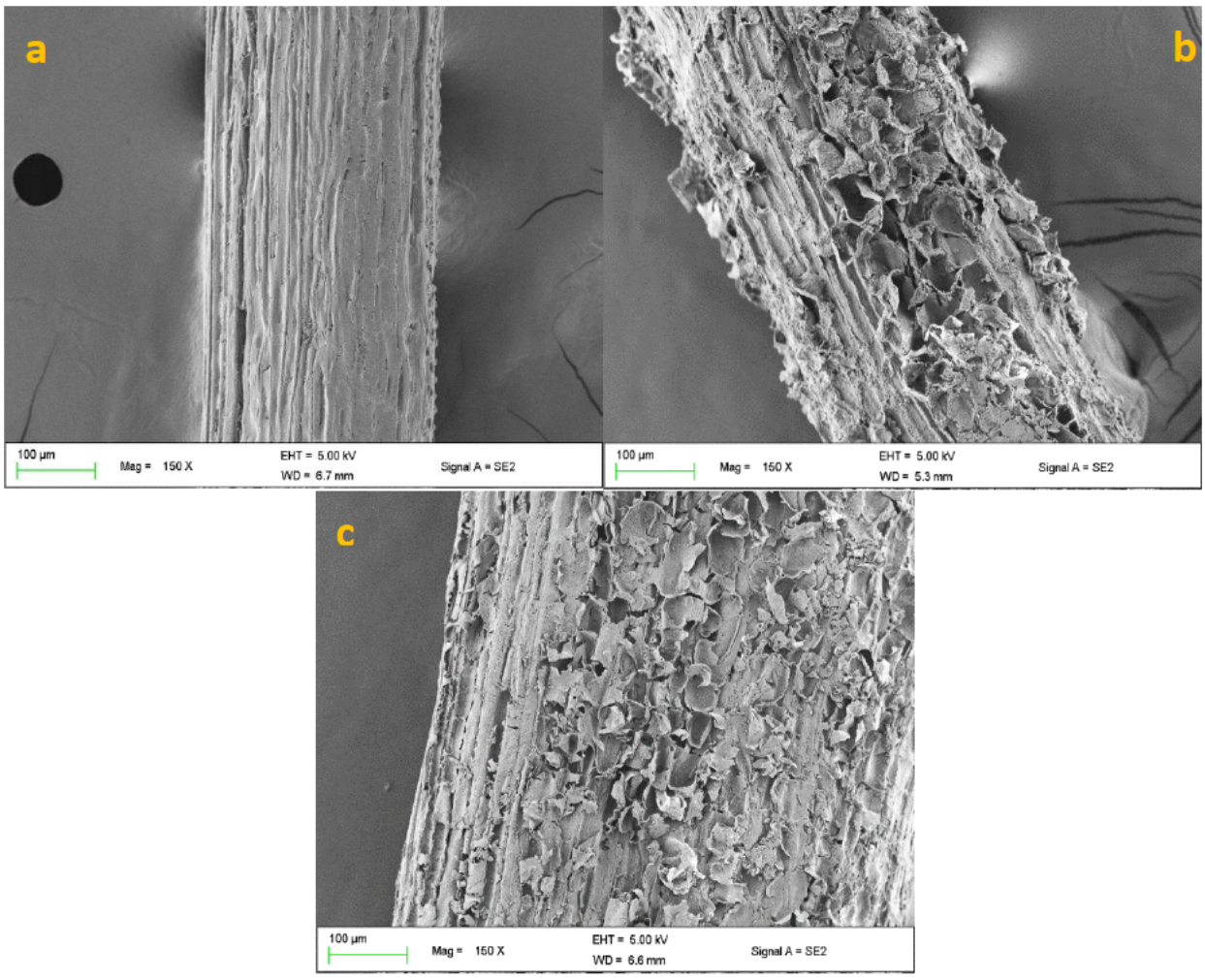


Fig. 7.1 SEM of untreated and biografted coconut fibres: **(a)** coconut fibre **(b)** coconut fibre modified with catechol (CF-Catechol) **(c)** coconut fibre modified with pyrogallol (CF-Pyrogallol)

7.3.1.2 FTIR spectra

Fig. 7.2 shows the FTIR spectra of untreated and grafted coconut fibres. The stretching peak at 3400 cm^{-1} is a characteristic peak for the -OH group present in coconut fibres which seems to shift towards lower wavenumbers (3310 cm^{-1} for CF-Cat; catechol grafted coconut fibres and CF-Pyr; pyrogallol grafted coconut fibres) in the biografted counterpart. The stretching peak at 3400 cm^{-1} also appears to be narrower and of a higher relative transmittance, in case of biografted coconut fibres. The intensity change of -OH groups can be due to the weakening of hydrogen bond which could be due to the formation of new bonds (Thakur *et al.*, 2015; Thakur *et al.*, 2016). A common band at 2927 cm^{-1} (appearing in both untreated and grafted coconut fibres) corresponds to C-H stretching vibrations (Dong *et al.*, 2014; Thakur *et al.*, 2015). The appearance of a new band at 1465 cm^{-1} can be attributed to the phenyl group of phenolic compounds (Aljawish *et al.*, 2012). In addition, in biografted fibres, bands in the range of 1400 and 1500 cm^{-1} can be attributed to an aromatic skeleton, which seems to be absent in untreated coconut fibres. Bands at 850 cm^{-1} and 600 cm^{-1} can be attributed to out of plane C-H stretching (Thakur *et al.*, 2015). The above results therefore confirmed the grafting of allelochemicals onto coconut fibres.

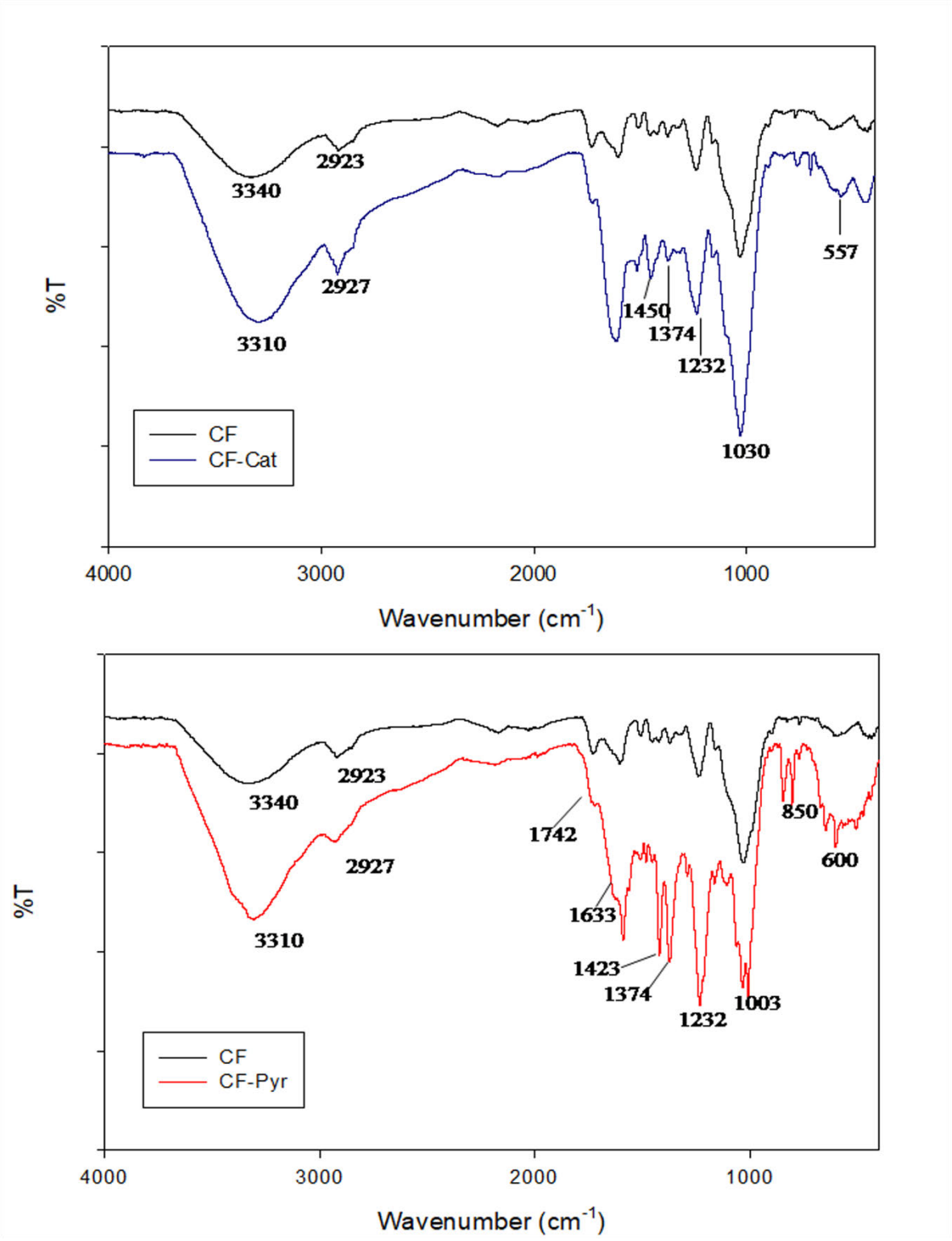


Fig. 7.2 FTIR spectra of untreated and biografted coconut fibres

7.3.2 Properties of untreated and grafted coconut fibres

7.3.2.1 Thermogravimetric analysis of untreated and grafted coconut fibres

Fig. 7.3 presents the thermal behaviour of untreated and functionalised coconut fibres. Both untreated and functionalised fibres showed three stages of decomposition. However, the functionalised fibres showed thermal behaviour which is different to that of the untreated coconut fibres. Initial weight loss or the first stage decomposition remains almost the same for treated and untreated materials, viz 6.7, 6.3 and 5.5% weight loss for CF, CF-Cat and CF-Pyr, respectively, at a temperature of 224°C. This first stage decomposition is due to evaporation and volatilisation processes. For untreated CF, maximum weight loss of 54.6% was observed in the range of 224-338 °C during the second stage of decomposition. However, in case of CF-Cat and CF-Pyr, 47.3 and 52.14% of weight loss was observed at 224-362°C and 224-360°C, respectively. It is worth noting that weight loss percentage in second stage decomposition for functionalised fibres is less as compared to its untreated CF counterparts even at extended temperature ranges (up to 360°C). This could be explained by thermal stability gained by means of functionalisation. In addition, at a temperature range of 224-338 °C, the weight loss is only 47.3% (7% less than CF control) and 41.45% (13% less than CF control) for CF-Cat and CF-Pyr, respectively. The second stage decomposition depicts the breakdown of the cellulose part of the coconut fibres. The final and the third stage decomposition led to a 38.2% weight loss in control fibres at a range of 338-505°C. The maximum weight loss of the functionalised fibres was 51.3% at the range of 338-503°C (for CF-Cat) and 48% weight loss at a range of 338-798 °C (CF-Pyr). The final stage marks the degradation of covalent bonds between lignin and the phenolic compound (grafted) (Thakur *et al.*, 2015). Weight loss at this stage for functionalised fibres is less than the untreated coconut fibres indicating that functionalisation of coconut fibres

had led to its improved thermostability. A shift to the right in the thermal curve of control and modified CF is indicative of improved thermal stability **(Fig. 7.3)**.

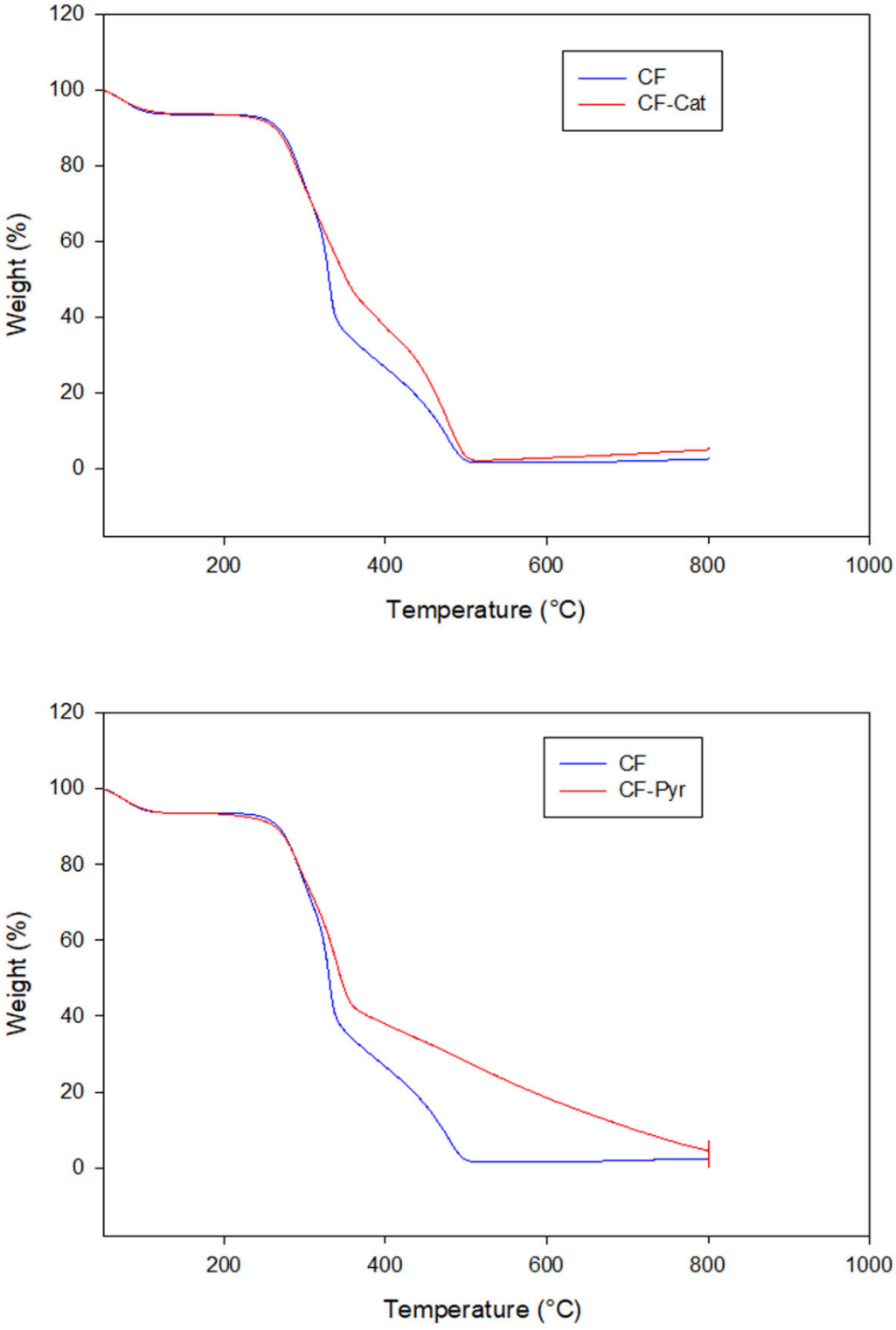


Fig. 7.3 Thermogravimetric analysis of untreated and biografted coconut fibres

7.3.3 Characterisation of functionalised chitosan films

7.3.3.1 SEM

SEM images of ungrafted and grafted chitosan films show an obvious difference in the surface properties after functionalisation **(Fig. 7.4)**. Control CHS film appears to have a smooth and continuous surface with grooves in between, while heterogeneously grafted chitosan films appear to have a coarse surface with irregular flakes of different sizes **(Fig. 7.4)**. However, homogenously grafted chitosan films appeared to have a dense and rough surface as compared to its ungrafted counterpart. The functionalisation process seemed to have increased the heterogeneity as well as roughness of the surface of chitosan films. This is consistent with the another report on functionalised chitosan (Aljawish *et al.*, 2016). However, the surface of homogenously grafted film was more uniform when compared to the heterogeneously grafted films.

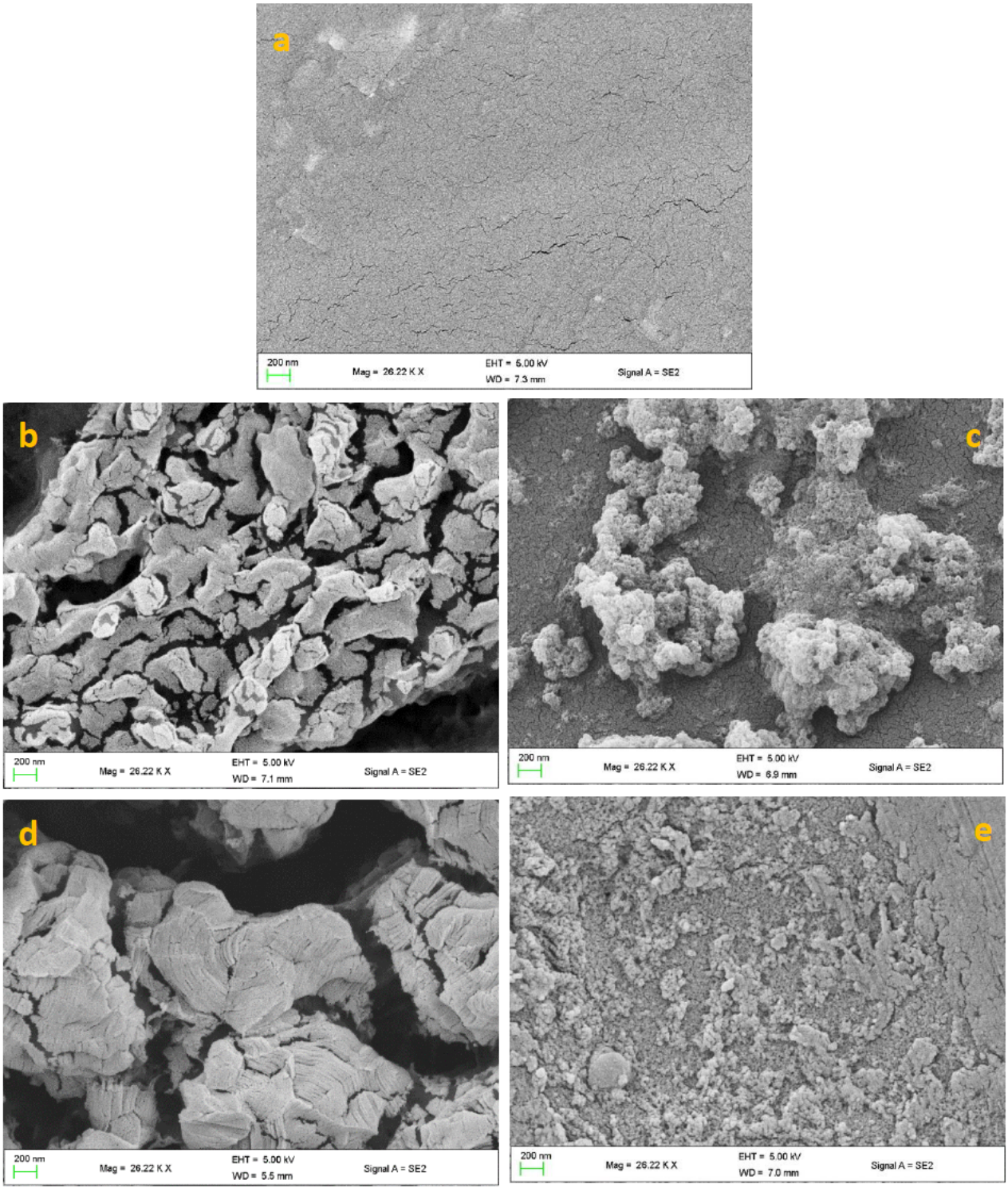


Fig.7.4 SEM of untreated chitosan and biografted chitosan: **(a)** chitosan; CHS **(b)** & **(d)** homogenously grafted catechol and pyrogallol **(c)** & **(e)** heterogeneously grafted catechol and pyrogallol

7.3.3.2 FTIR

Fig. 7.5 shows the FTIR spectra of unmodified and functionalised CHS films at pH 4.5. Untreated CHS film (control) shows an intense absorption band in the range of 3200-3500 cm^{-1}, attributed to -OH and -NH stretching of a polysaccharide molecule (Bożiĉ *et al.*, 2012). Stretching vibrations at 1650, 1550 and 1320 cm^{-1} are attributed to amide I (C-O), II (N-H), and III (C-N) of the residual N-acetyl groups, respectively, indicating incomplete deacetylation of chitosan (Rui *et al.*, 2017). However, the broad peak at 3240 cm^{-1} (CHS-Cat) and 3414 cm^{-1} (CHS-Pyr) shifts to lower frequency in functionalised chitosan films as compared to the control. In addition, changes in the 3300-3500 cm^{-1} and 1590-1650 cm^{-1} can be attributed to -NH stretching and -NH bending, respectively (Chen *et al.*, 2000). Further, an absorption decrease is observed at 1320 cm^{-1}, 1380 cm^{-1} (-NH bending of the glucosamine unit) and 1420 cm^{-1} (symmetric $-NH_3^+$ bending region) in case of functionalised films. This indicates loss of $-NH_3^+$ groups which can be explained by the covalent reaction between the laccase-catalysed products of allelochemicals and amino groups of chitosan (Aljawish *et al.*, 2012). Changes in the region of 1000-1500 cm^{-1} could be due to the aromatic C-O stretching of the catechol and pyrogallol, which is consistent with previous reports (Sousa *et al.*, 2009; Aljawish *et al.*, 2012).

In addition, -NH bending at 1530 cm^{-1} is also supportive of a Michael addition-type reaction. Also, a decrease (in case of CHS-Cat) and complete disappearance (in case of CHS-Pyr) of the absorption peak at 1546 cm^{-1} can be attributed to a decrease in $-NH_2$ group content, which indicates reaction of chitosan's amino groups with the phenolics, forming a Schiff base. The results are consistent with those from a previous report (Jin *et al.*, 2009). The shift from 1603 cm^{-1} (N-H of amine I groups) down to 1560 cm^{-1} (N-H of amine II groups) is also noteworthy, as this indicates change in the local environment of nitrogen atoms (Crouvisier-Urion *et al.*, 2019).

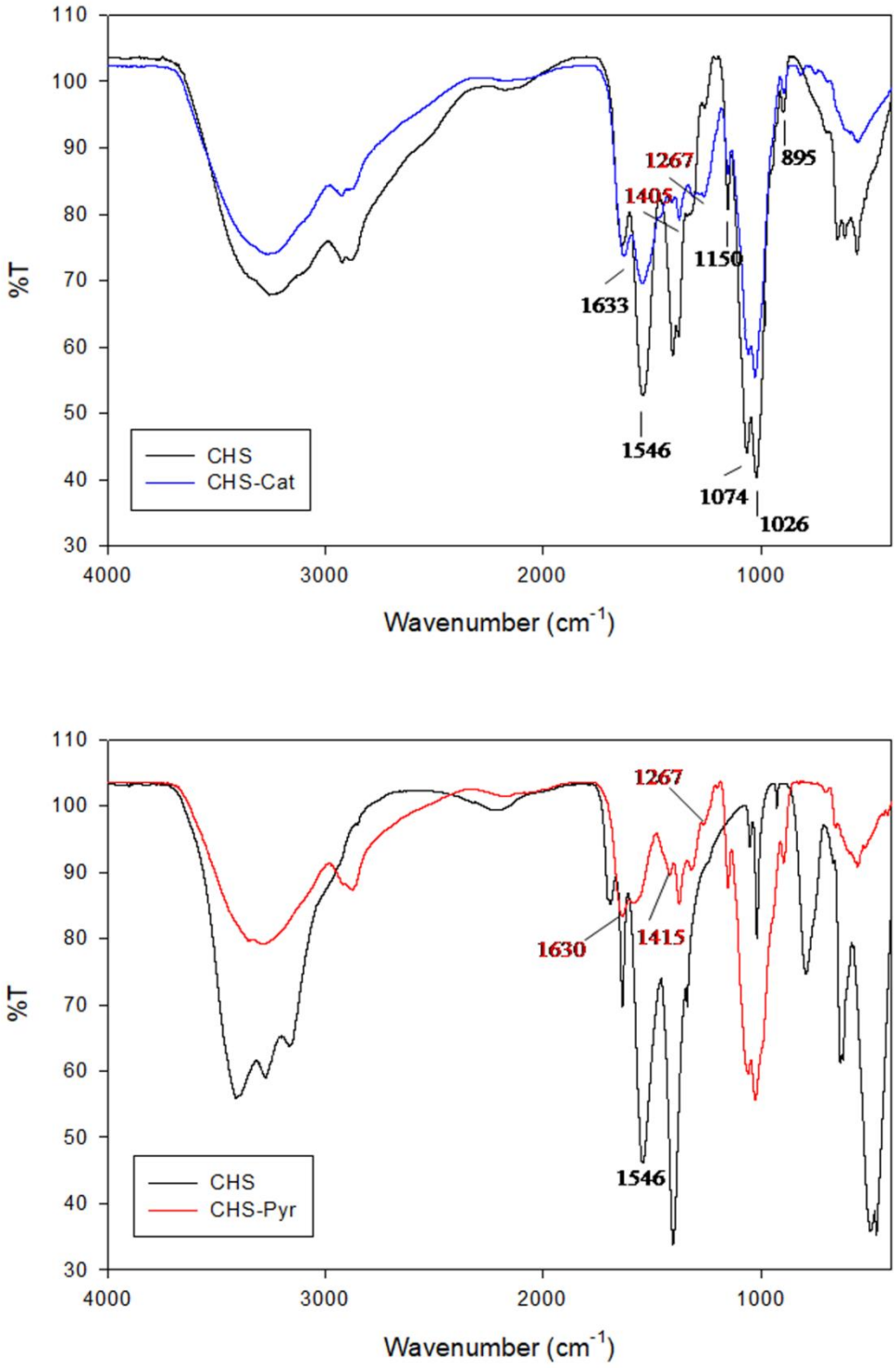


Fig.7.5 FTIR spectra of untreated and functionalised chitosan films **(a)** untreated CHS and CHS-Cat **(b)** untreated CHS and CHS-Pyr.

Significant features including shouldering of 1630 cm^{-1} band, is a characteristic feature of C-N stretching of imines, further supporting the evidence of a Schiff-base reaction. Catechol and pyrogallol functionalised films showed the expected signals associated with the polysaccharide backbone and the presence of the catecholic motif including the aryl (1405 cm^{-1}) and phenolic alcohol bands (1267 cm^{-1}). This is consistent with previous reports (Zvarec *et al.*, 2013; Ryu *et al.*, 2015). The appearance of new absorbance peaks provides additional evidence that the allelochemicals had been successfully grafted onto chitosan.

7.3.3.3 UV/Vis spectroscopy

Characterisation of chitosan and its derivatives were also performed by UV/Vis spectroscopy in order to provide chemical evidence of any specific linkage between the laccase-oxidised phenolic compounds and the amino groups of chitosan. Significant changes in the UV/Vis spectra of test (CHS Pyr + rSLAC; CHS + Cat + rSLAC) and control reactions (without rSLAC) were observed at pH 6 **(Fig. 7.6)**.

The high absorption peak at the start of the spectra (below 300 nm) (**Fig.7.6)** could be attributed to the π system of the benzene ring (Zheng *et al.*, 2018). In addition, UV absorption at 270 nm is a characteristic of catechol (Qiao *et al.*, 2012; *et al*., 201Ryu5; Qiu *et al.*, 2016). Appearance of a peak at around 380 nm is indicative of a reaction between *o*-quinone (or products of laccase-catalysed oxidation of catechol or pyrogallol) and amino groups of chitosan (Bożiĉ *et al*., 2012). The constant increase in the intensity of peaks at 380 nm and 450 nm was also observed by Sousa *et al*. (2009) along with the visual browning of the reaction. On the contrary, control reactions without laccase (CHS+Cat or CHS+Pyr) did not change colour after incubation **(result not shown**). The phenomenon of visual browning has also been observed in other reports (Chen *et al.*, 2000; Widsten *et al.*, 2010; Qiu *et al.*, 2016). In addition, control

reactions without rSLAC didn't show any new peaks even after an extended reaction time of 60 min. These observations indicate that the presence of enzyme is necessary for the oxidation of the allelochemicals and subsequent grafting of oxidised catechol or pyrogallol onto chitosan. Conversely, UV/Vis absorption peaks of chitosan derivatives showed peaks at the higher wavelengths (or visible regions) which increased with time. This could be explained by the progressive crosslinking between the quinones and amine with increase in incubation time (Qiu *et al.*, 2016). Also, an increase in absorbance at wavelengths higher than 500 nm can be associated with Michael type addition with respect to catechol and amines (Yang *et al.*, 2016).

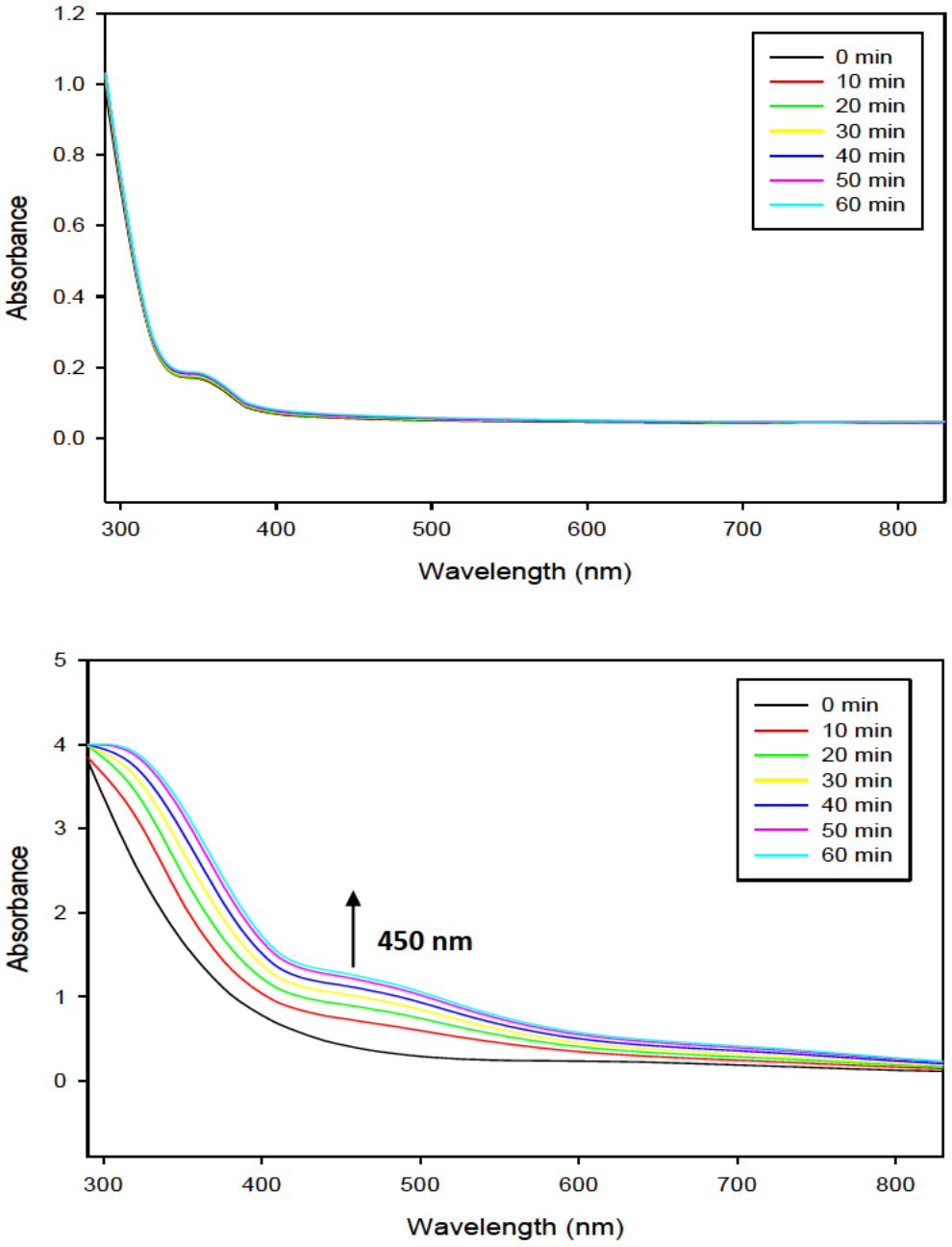


Fig. 7.6 a) UV/Vis spectra of during laccase-catalysed coupling of catechol to chitosan / (reaction time 0-60 min).

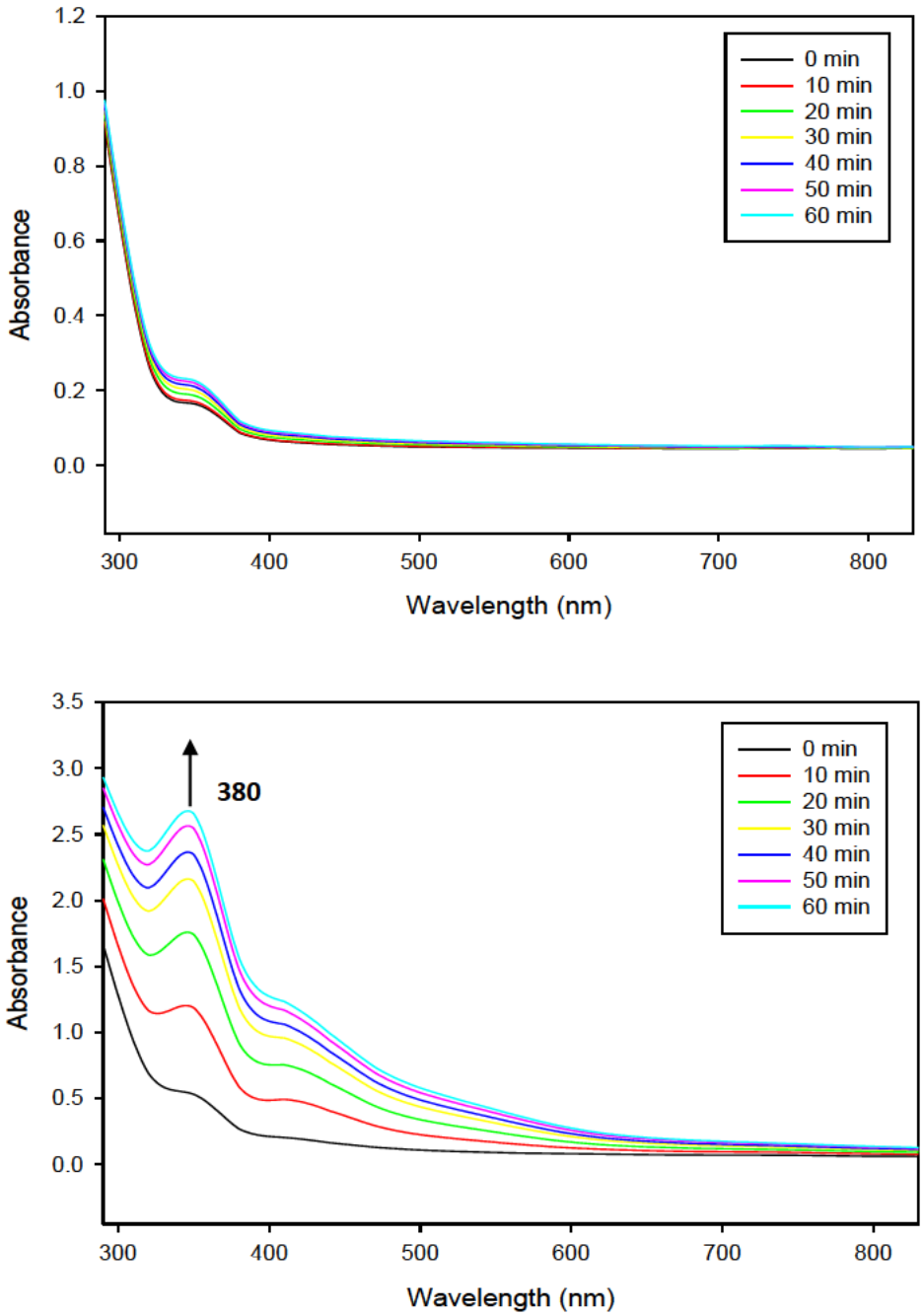


Fig. 7.6 b) UV/Vis spectra of laccase-catalysed coupling of pyrogallol to chitosan (reaction time 0-60 min)

7.3.4. Antibacterial properties of untreated and functionalised coconut fibres

The antibacterial properties of functionalised materials (CF-Cat/CHS-Cat and CF-Pyr/CHS-Pyr) were tested against a Gram positive (*S. aureus*) and Gram-negative (*E. coli*) bacterium. Catechol and pyrogallol (allelochemicals) are well known for their antibacterial activities. Ungrafted chitosan films as well as coconut fibres showed no antibacterial activity against the strains tested **(Fig. 7.7)**. On the other hand, the grafted coconut fibres exhibited enhanced antibacterial effects as compared to the untreated fibres. Greater colony forming units (CFU) reductions were observed against *E. coli* as compared to *S. aureus*. A 60 and 52% growth inhibition with CHS-Cat was observed against *E. coli* and *S. aureus*, respectively. However, chitosan functionalised with pyrogallol (CHS-Pyr) exhibited only 35 and 10% growth inhibition against *E. coli* and *S. aureus*, respectively. Coconut fibres grafted with catechol (CF-Cat) and pyrogallol (CF-Pyr) exhibited greater growth inhibition 34-59% against *E. coli* and *S. aureus*, respectively, in comparison to their functionalised chitosan counterparts. Antibacterial activity against *E. coli* can be attributed to the disturbance in the divalent cations (present on the outer membrane of *E. coli*) caused by the anionic ions present in the grafted biomaterials. It is well known that lipopolysaccharides and protein make up the outer membrane of *E. coli*. The divalent cations and electrostatic interactions which maintain the stability of the outer membrane are disturbed when in contact with phenolic compounds (Thakur *et al.*, 2015) Furthermore, the antibacterial effects can be attributed to the phenoxy hydroxyl group (-OH group) and the delocalised electron system (Thakur *et al.*, 2015) of the allelochemicals, acting as a proton exchanger that can alter the pH gradient of the cytoplasmic membrane. This ultimately results in collapsing of the proton-motive force which in turns depletes the ATP pool, eventually leading to the death of *E. coli* (Bożiĉ *et al*., 2012). On the contrary, rupturing of the thick peptidoglycan layer of *S. aureus* is tougher which explains the decreased antibacterial activity of grafted materials against this bacterium. However, compared to the

pyrogallol-grafted materials, catechol-grafted materials showed greater performance in terms of antibacterial activity.

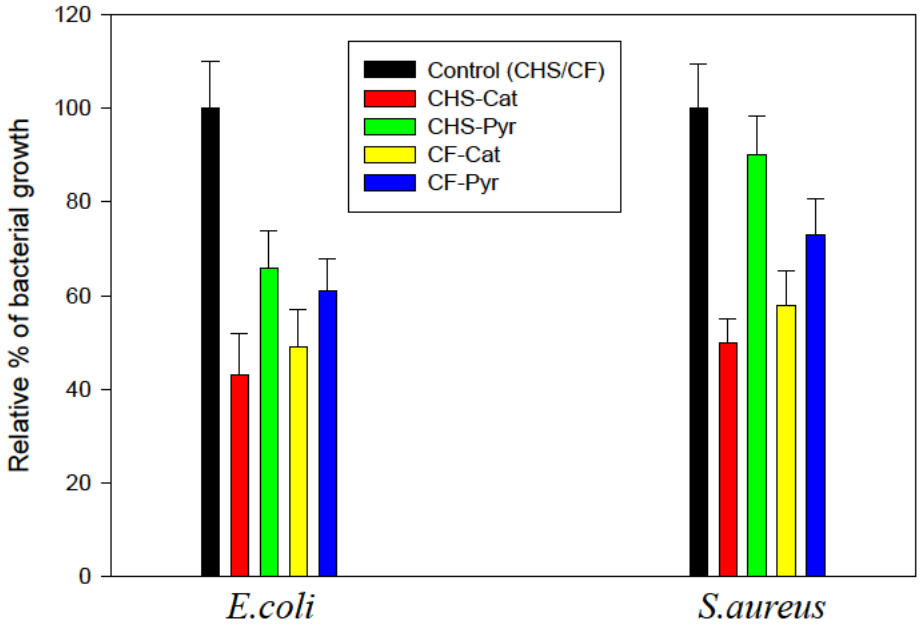


Fig. 7.7 Relative percentage of bacterial growth after inoculation of colony forming units (CFUs) on ungrafted and grafted chitosan films and coconut fibres. Error bars indicate standard deviation of three independent experiments.

7.4 Conclusions

In the present study, the SLAC has been used for the first time as a catalyst for the grafting of antimicrobial compounds, catechol and pyrogallol onto two widely available natural polymers, coconut fibres and chitosan. FTIR, SEM, TGA and UV/Vis analyses provided evidence of SLAC-mediated grafting of the functional molecules onto the coconut fibres and chitosan. The functionalised materials showed antibacterial activities against *E. coli* and *S. aureus*. Based on the results obtained, it can be envisioned that the allelochemical-functionalised derivatives of chitosan and coconut fibres can open up new avenues for applications in hygienic packaging

material (functionalised chitosan) and in the development of composites (functionalised coconut fibres).

CHAPTER 8

General Discussion

In this thesis, the small laccase (SLAC) from *Streptomyces coelicolor* A3(2) was engineered, immobilised and expressed in a recombinant host for improving its catalytic efficiency, stability and production levels. The first part of this section discusses the engineering aspect of SLAC (Chapter 3), the second part discusses the secretory expression of SLAC in *Pichia pastoris* (Chapter 4), while the third part explores the use of dual promoter system to enhance the production levels of SLAC in *Pichia pastoris* (Chapter 5). The fourth part focuses on the immobilisation of SLAC (Chapter 6) followed by the application of SLAC in the surface functionalisation of coconut fibres and chitosan with allelochemicals (Chapter 7). In addition, potential application of the recombinant small laccase (rSLAC) was also investigated in dye decolorization (Chapter 4), degradation of antibiotics (Chapter 4) and phenolic pollutant degradation (Chapter 5).

Contamination of groundwater reservoirs and soil by textile dye effluents, phenolic pollutants, and the presence of antibiotics, pose a major threat to global environmental health. These pollutants are toxic, carcinogenic, and/or mutagenic; some of them (such as phenol and halogenated phenols) are even listed as “priority pollutants”. Physio-chemical treatments are often complicated, expensive, and generate sludge, which require additional treatment steps. Biological treatment seems to be an environmentally friendly and inexpensive alternative (Koschorreck *et al.*, 2017). Emphasis has been placed on biological treatments which has been classified into: (i) whole-cell; and (ii) biocatalysts (Woodley *et al.*, 2013). It is worth noting that lack of nutrients in toxic environment impedes microbial growth, thereby delaying any natural remediation processes that may take place (Sharma and Philip, 2014). An ideal bioremediation process must be able to cope with the extreme environment and should be

independent of the availability of nutrients (Harms, Schlosser and Wick, 2011). Therefore, biocatalytic processes are usually preferred.

In this regard, white rot fungi and their extracellular enzymes have been intensively investigated for bioremediation purposes (Koschorreck *et al.*, 2017). Among them, laccase, owing to its broad substrate specificity could prove to be an ideal biocatalyst. Currently, laccases from fungal origin have been investigated for most applications. However, they operate at mildly acidic pH and a temperature range of 30-55 °C (Koschorreck *et al.*, 2017). It is worth noting that the pH of hospital and industrial wastewater is generally in the neutral to alkaline range (Cruz-Morató *et al.*, 2014; Ouyang and Zhao, 2019). This could lead to inactivation of fungal laccases (Ouyang and Zhao, 2019) as ligation of -OH groups to the tri-nuclear T2/T3 cluster prevents electron transfer from the T1 copper to the T2/T3 cluster (Yadav *et al.*, 2018). Therefore, bacterial laccases such as SLAC seem to be ideal biocatalysts for these bioremediation processes, as they are stable at alkaline pH and are thermostable (Chandra and Chowdhary, 2015).

Despite the favourable attributes of SLAC, the significant obstacle is the lack of sufficient enzyme stocks (Singh *et al.*, 2011) along with its low enzyme activity and catalytic efficiency. Naturally, enzymes are not well-suited for their use under harsh industrial conditions. Therefore, it becomes imperative to make desirable changes in the enzyme which can be achieved by protein engineering approaches. Protein engineering enables the modification of an enzyme at the sequence level and can bring the desired change in terms of solubility, selectivity, stability, activity, catalytic properties such as tolerance towards solvents, temperature or extreme pH values. Broadly, protein engineering approaches can be divided into two categories (1) rational design, and (2) random mutagenesis (Damián-Almazo and

Saab-Rincón, 2013). In rational design, target sites are identified based on the structural information of the protein to obtain the modified protein (Yuan *et al.,* 2017). To aid in the rational design, computational predictive algorithms can be used to predict promising target sites (Damián-Almazo and Saab-Rincón, 2013). Furthermore, it has been reported that single mutations, when combined has an additive effect (Rodriguez *et al.*, 2000; Zhou *et al.*, 2018), is also seemingly a straightforward approach. Industrially desirable characteristics of SLAC (high thermostability, activity at high temperature, activity under alkaline conditions, and stability in the presence of inhibitors) prompted us to improve its catalytic performance. In the present study, we combined mutations from different mutagenesis studies which targeted to improve the activity and turnover number of SLAC. Combining the mutations led to the identification of two evolved mutants, Y230A/V290N and Y229A/M198G, with improvement in substrate affinity and increment in catalytic efficiency, respectively. Overall, the results demonstrated that site-directed mutagenesis (SDM) could overcome some limitations of small laccases and result in the generation of an evolved SLAC with potential industrial application.

The SLAC gene from *S. coelicolor* A3(2) consists of 1029 nucleotides (translates into 343 amino acids) and codes for a highly thermostable enzyme. This enzyme has been characterised (Machczynski *et al.*, 2004) and shows tremendous potential in dye decolorization (Dubé *et al*., 2008). Furthermore, homologous production of SLAC in *Streptomyces lividans* resulted in a production level of 350 mg/L (Dubé *et al.*, 2008). Suzuki *et al*. (2003) also reported its cloning and expression in *Escherichia coli* (most preferred recombinant host) leading to a production yield of 30 mg/L. However, overexpression in this host leads to intracellular aggregation (Dubé *et al.*, 2008), which requires additional steps in downstream processing which could lead to an increase in production costs. Extracellular expression could be a desirable alternative in this regard (Ahmad *et al*., 2014). Therefore, in this study, the problem was resolved by cloning of

SLAC (recombinant SLAC; rSLAC) in pPICZαA for expression in *Pichia pastoris* GS115. SLAC expressed in *P. pastoris* showed an extracellular production level of 500 U/L (after optimisation of methanol concentration and medium composition) and exhibited higher catalytic efficiency than CotA laccase, a well-studied bacterial laccase (Fan *et al.*, 2015; Wang *et al.*, 2015).

Constitutive expression in a pGAPZαA vector was also explored for enzyme expression. A constitutive promoter (P_{GAP}) involves easy process handling as it negates the need to use the hazardous inducer (i.e. methanol) and ensures continuous transcription of the cloned gene (He *et al.*, 2015). However, low expression levels were observed in constitutive expression. Therefore, the inducible (P_{AOX}) and constitutive (P_{GAP}) promoters were combined to generate a dual promoter vector. *Pichia pastoris* GS115 harbouring a dual promoter construct resulted in the enhancement of expression levels by 2.4-fold compared to rSLAC-AOX and 5.1-fold compared to rSLAC-GAP. Since every promoter has its own specific requirements in terms of carbon source (He *et al.*, 2015), an investigation to find a balanced ratio of methanol:glycerol was also carried out as residual glycerol could completely shut off the AOX promoter (Parashar and Satyanarayana, 2016). A stoichometric ratio of 9:1 further enhanced the expression levels of rSLAC-AOX-GAP by 1.5-fold when compared to induction by methanol alone.

Free enzymes are associated with several drawbacks such as large consumption, difficult separation and recycling which are obstacles for application at industrial scale (Fernandes *et al.*, 2017; Ranjan *et al.*, 2017). Immobilisation of enzymes could overcome the challenges associated with free enzymes (Chen *et al.*, 2015). In this regard, several techniques such as adsorption, cross-linking, encapsulation, entrapment and covalent binding have been employed (Sheldon, 2007). Covalent binding between the biocatalyst and the support matrix is generally

preferred for industrial applications (DiCosimo *et al.*, 2013; Barbosa *et al.*, 2015; Hosseini *et al.*, 2018). The choice of matrix also plays a key role in catalysis (Zdarta *et al.*, 2018). Nanomaterials have been gaining attention in recent years due to the their small size, high surface to volume ratio, thermostability, aqueous dispersibility and chemical inertness (Patila *et al.*, 2016). In this study the immobilisation of rSLAC onto magnetic nanoparticles (MNP) was investigated. The rSLAC immobilised onto MNPs displayed a 73% residual activity after 10 cycles and showed remarkable stability in the presence of metal ions which could be advantageous, as heavy metal ions are usually found in industrial wastewater (Zeng *et al.*, 2017). In addition, the immobilised rSLAC showed enhanced resistance towards organic solvents. The results suggest that immobilisation of rSLAC could open new avenues for the application at large-scale, especially in bioremediation. Development of a reusable enzyme system could be beneficial especially when the high cost of production of the enzymes is taken into account (Chhabra *et al.*, 2015). One other advantage of enzyme immobilisation, besides maintaining the enzyme in the system, reusability and improved enzyme stability, is process control.

The final goal was to apply the rSLAC in dye decolourisation, degradation of phenolic pollutants, antibiotic degradation and surface functionalisation.

The evolved mutant (Y230A/V290N) displayed remarkable potential in the decolourisation of a broad range of dyes such as Methyl red (MR; 58.29%), Malachite Green (MG; 66.99%), Brilliant Green AR (BGAR; 50.11%), Remazol Brilliant Blue R (RBBR; 62.29%) and Reactive Orange 16 (RO16; 44.64%). On the contrary, wild-type SLAC (WT-SLAC) could only decolourise 28.43, 47.15, 31.13, 38.50 and 34.20 % of MR, MG, BGAR, RBBR and RO16, respectively. The increase in decolourisation potential could be related to the low K_m value of

Y230A/V290N (75.5% lower than the WT-SLAC for ABTS). A low K_m value denotes higher substrate affinity. Furthermore, another double mutant (Y229A/M198G) exhibiting superior catalytic efficiency and was able to completely decolourise 50 mg/L of methyl red. However, Y230A/V290N and WT-SLAC could only decolourise 58.12% and 28.35% of methyl red, respectively.

Investigation of the potential of rSLAC to degrade commonly used broad spectrum antibiotics ciprofloxacin (CIP) and tetracycline (TC) revealed that rSLAC alone could degrade 16% and 74% of 5 mg/L of CIP and TC, respectively. Difference in the degradation efficiency of these antibiotics could be attributed to the difference in their structure, where TC has a phenolic structure which is not present in CIP. However, inclusion of acetosyringone (AS), a natural mediator, enhanced the rate of degradation of both antibiotics. Only 0.4 U rSLAC degraded ≥95% of 5 mg/L of CIP and 200 mg/L of TC, indicating the high catalytic efficiency of rSLAC. Degradation of CIP improved with increase in pH. For example, only 20% of CIP was degraded at pH 4 while, maximum degradation (approximately 95%) was observed at pH 8. On the other hand, pH didn't seem to have a significant effect on the degradation rate of TC. The results have positive implications for bioremediation as the reported pH of urban wastewater and hospital effluents is in the alkaline range (Cruz-Morató *et al.*, 2013, 2014). Advanced oxidation processes are incapable of completely mineralising contaminants, and could lead to the generation of even more toxic products than the contaminant itself (Gou *et al.*, 2014). Therefore, the results obtained from rSLAC-mediated degradation of dyes, phenolic pollutants and broad-spectrum antibiotics seems promising, especially as degradation of phenolic pollutants reduced the toxicity of the compounds.

Finally, rSLAC was employed as radical initiator to facilitate the covalent grafting of antimicrobial compounds, catechol and pyrogallol onto lignin moieties and amino groups of coconut fibres and chitosan, respectively. Formation of a covalent bond between phenolic compound and biopolymer was verified by several techniques such as FT-IR and UV-Vis spectrophotometry. The grafted conjugates showed good antibacterial activity against *E. coli* ATCC 25922 and *Staphylococcus aureus*. 29213 The results suggest that the rSLAC can be exploited for biografting of fibres and chitosan films into value-added products.

In conclusion, strategies have been developed for enhancing the potential of SLAC as an industrial biocatalyst. Replacement of residues in the substrate binding pocket resulted in changes in its specificity and catalytic efficiency. Expression in *P. pastoris* enhanced expression level and immobilisation on nanoscale material enhanced its storage stability and reusability. Overall, the strategies developed in this study will provide the foundation for the widespread application of SLAC from *Streptomyces coelicolor*.

CHAPTER 9

Conclusions and Recommendations

There has been an increasing interest towards bacterial laccases owing to its industrially favourable characteristics. However, there has been some drawbacks associated with bacterial laccases such as low catalytic efficiency and expression levels. Additionally, lack of reusability of free enzymes is another drawback associated when it comes to apply a biocatalyst at an industrial level. Therefore, this study investigated the engineering of small laccase (from *Streptomyces coelicolor*) for its application in biocatalysis and surface functionalisation

The specific objectives of this study were as follows:

1. To enhance the activity of SLAC by site directed mutagenesis.
2. To clone and express the SLAC gene in *P. pastoris* for secretory expression using a single and double promoter system.
3. To immobilise the SLAC on magnetic nanoparticles
4. To apply the SLAC in bioremediation and surface functionalisation.

Based on the research performed, the following conclusions can be made:

- Composite mutants (Y230A/V290N and Y229A/M198G) generated via site-directed mutagenesis showed a better substrate specificity (low K_m) than the wild-type small laccase (WT-SLAC). One composite mutation (Y229A/M198G) showed improved catalytic efficiency (k_{cat}/K_m) when compared with WT-SLAC. The Y229A/M198G mutant showed complete decolourisation of 50 mg/L of methyl red in comparison to

only 58.29% and 28.43% decolourisation by Y230A/V290N and WT-SLAC respectively.

- The SLAC was cloned for the first time in *Pichia pastoris* for secretory expression. The SLAC gene comprising of 1029 nucleotides was cloned in pPICZαA. The pPICZαA vector contains an AOX1 promoter for the tight regulation and methanol induced expression of the gene cloned under it. Optimisation of expression conditions of the recombinant strain harbouring *pPICZαA-SLAC* enhanced SLAC expression. YP medium was found to be the best medium and 2% (v/v/) methanol was found to be most effective inducer concentration enhancing rSLAC production to approximately 500 ± 10 U/L.

- The SLAC gene was also cloned into a constitutive expression vector (pGAPZαA). A low expression level of 235 U/L was achieved with the GAP promoter. Therefore, a dual promoter (AOX and GAP) system for expression of SLAC was developed. Profiling for cell growth and rSLAC production revealed maximum rSLAC expression (1200 U/L) after the 5^{th} day of cultivation which was 2.4-fold higher than the expression achieved with a single promoter system. In addition, there was a significant increase in cell growth of the recombinant *Pichia* strain with dual promoters. Highest rSLAC activity was recorded at pH 7 and began to drop at acidic pH.

- rSLAC was successfully immobilised on modified magnetic nanoparticles (MNPs). Stability analysis revealed its excellent thermostability, pH stability, stability towards heavy metals and organic solvents, storage stability and reusability. Approximately 73% of its initial activity was retained after 10 catalytic cycles.

- SLAC degraded ≥98% of 80 mg/L phenol, 4-chlorophenol (4-CP) and 4-fluorophenol (4-FP) over a wide pH range (6-8) in the presence of a natural mediator, acetosyringone (AS), within 2 h of reaction time. Increase in pH led to an increase in degradation percentage for all three phenolic pollutants. Toxicity of phenol, 4-CP and 4-FP was reduced by 90, 60 and 55%, respectively.

- In the presence of acetosyringone, SLAC (0.4 U) degraded ≥95% of 5 mg/L of ciprofloxacin (CIP) and ≥95% of 200 mg/L tetracycline (TC) within 6h. The toxicity of the antibiotics was reduced by 100% and 48%, respectively.

- In addition, two biological materials (chitosan and coconut fibres) have been engineered to enhance antimicrobial functionality using SLAC as catalyst. The recombinant rSLAC oxidised functional molecules to corresponding radicals which reacted with the lignin moieties and amino groups of the coconut fibres and chitosan, respectively. Modified coconut fibres and chitosan showed increased antimicrobial activity.

Recommendations:

Combining immobilised rSLAC and a mediator could prove to be a promising tool in clean-up of wastewater effluents contaminated with dyes, phenolic pollutants and antibiotics. Results obtained with rSLAC alone (in case of dye degradation) and rSLAC-AS (in degradation of phenolic pollutants and antibiotics) were comparative to those of the advanced treatment technologies such as photodegradation and oxidation, but have the advantage of operating under mild reaction conditions and having fewer toxic side reactions. Furthermore, SLAC-mediated functionalisation presents an eco-friendly alternative approach to the existing physio-

chemical processes. Based on the results obtained, it can be envisioned that these allelochemical functionalised derivatives of chitosan and coconut fibres can open up new avenues for applications in hygienic packaging material (functionalised chitosan) and in the development of composites (functionalised coconut fibres).

Modification of catalytic properties by site-directed mutagenesis as well as the immobilisation and expression under dual promoter system look promising for potential industrial application of the enzyme. However, the major challenge could be the scale of reactions.

Based on the research performed in this study, further research could be focused on:

i) rSLAC production can be performed in pilot scale fermenters with the optimized conditions in shake flasks in order to assess the scalability of the developed strategies.

ii) Investigating the applicability of rSLAC in degradation of the tested contaminants in the simulated wastewater conditions.

iii) Simultaneous removal of antibiotics (belonging to different categories) can be attempted as more than one type of antibiotic can exist in environmental matrices of hospital wastewater effluents.

iv) Analytical methods such as mass spectrometry and NMR analysis could be performed to characterize the metabolites and gain a clear insight into the possible mechanism involved during rSLAC-mediated degradation of the investigated compounds.

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Secretory expression of recombinant small laccase from *Streptomyces coelicolor* A3(2) in *Pichia pastoris*

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ABSTRACT

This work reports for the first time the secretory expression of the small laccase (SLAC) from *Streptomyces coelicolor* A3(2) in *Pichia pastoris*. Using an AOX1 promoter and α factor as a secretion signal, the recombinant *P. pastoris* harbouring the laccase gene (*rSLAC*) produced high titres of extracellular laccase (500 ± 10 U/l), which were further increased seven fold by pre-incubation at 80 °C for 30 min. The enzyme (~38 kDa) had an optimum activity at 80 °C, but optimum pH varied with substrate used. K_m values for ABTS, SGZ and 2,6-DMP were 142.85 μM, 10 μM and 54.55 μM and the corresponding k_{cat} values were 60.6 s^{-1}, 25.36 s^{-1} and 27.84 s^{-1}, respectively. The $t_{1/2}$ values of the rSLAC at 60 °C, 70 °C, 80 °C were 60 h, 32 h and 10 h, respectively. The enzyme deactivation energy (E_d) was 117.275 kJ/mol while ΔG, ΔH and ΔS for thermal inactivation of the rSLAC were all positive. The rSLAC decolourised more than 90% of Brilliant Blue G and Trypan Blue dye in 6 h without the addition of a mediator. High titres of SLAC expressed in *P. pastoris* enhance its potential for various industrial applications.

1. Introduction

Laccase is a multicopper oxidase, capable of catalysing one-electron oxidation of a wide range of substrates such as diphenols, methoxy-substituted monophenols, aromatic and aliphatic amines to generate radicals while concomitantly reducing molecular oxygen to water [1]. Laccases are widely distributed in nature; they have been isolated from fungi, plants, insects and bacteria [2] of which fungal laccases are well characterised [3]. Currently most applications are carried out with fungal laccases. However, applicability of fungal laccases is hindered in industrial processes which operate at harsh conditions of temperature, pressure or extremely alkaline pH [4,5]. Moreover, it is worth noting that fungal laccases show optimum activity at an acidic pH range and laccase catalysed oxidation efficiency is lowered with increase in pH due to OH^- ligation to the tri-nuclear T2/T3 cluster which prevents the internal transfer of electron from T1 copper to the T2/T3 cluster [6].

Bacterial laccases, such as the small laccase from *S. coelicolor* (SLAC), can be useful alternatives to fungal laccases. SLAC exhibits some desirable characteristics such as activity at high temperatures and alkaline pH [7], making it a potential candidate in biotechnological applications. However, low production yield by native microbial hosts [8] is a major bottleneck for commercial applications [9]. Consequently, a vast amount of research has focused on the search for simple heterologous expression systems, with *Escherichia coli* as the most frequently used expression system [10]. Features such as availability of established bacterial genetic tools and biotechnological processes, plasmid DNA stability and high transformation efficiency make *E. coli* the most sought after host system [10,11]. However, overexpression in *E. coli* is often limited by intracellular aggregation [12,13] leading to significantly low yields of soluble protein [14–17]. In addition, inclusion bodies are also difficult to purify thereby increasing production costs [10].

Extracellular expression could be a desirable alternative for expression of foreign proteins since it simplifies purification steps [18]. *P. pastoris* is usually the preferred host for the production of industrial enzymes [18]. It offers several advantages over other recombinant expression hosts such as ability to grow at high cell densities, low levels of native proteins, no antibiotic requirement once a stable integrant is obtained, and availability of expression vectors which utilise methanol as an inexpensive carbon source and inducer [19,20]. *P. pastoris* has been successfully used in express-

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Table 1
Primers used in this study; underlined sequence indicate the area where restriction sites were added to the primers.

Primers	Oligonucleotide Sequence (5′ → 3′)	Restriction site
SLAC F′	CGGAATTCATGGACAGGCGAGGCTTTAA	*Eco*RI
SLAC R′	CGTCTAGATAA GTGCTCGTGTTCGTGTGCGG	*Xba*I

GAATTC, restriction site for *Eco*RI; GGTACC, TCTAGA, restriction site for *Xba*I.

ing high yields of recombinant proteins through high cell density cultivation [21]. Hence, using an expression plasmid with α-factor signal sequence can prove to be useful in enhancing the production of SLAC.

In the current study, we explore heterologous expression of the SLAC gene from *S. coelicolor* in *P. pastoris*, to facilitate high level production of this enzyme. The recombinant protein was purified and characterised for its kinetic and thermodynamic parameters. In addition, the decolourisation ability of the rSLAC on Brilliant Blue G (Fig. S1A) and Trypan Blue (Fig. S1B), was investigated.

2. Materials and methods

2.1. Materials

2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), syringaldazine (SGZ), Brilliant Blue G, and Trypan Blue were purchased from Sigma-Aldrich (South Africa).

2.2. Microorganisms and media

A pET-20b(+) vector (Novagen) containing the gene coding for SLAC (pSLAC) (WP_003972284) was cloned into *E. coli* BL21(DE) cells and was kindly provided by Prof. Gerard Canters, Leiden Institute of Chemistry, University of Leiden, The Netherlands. *E. coli* DH5α used for the construction and routine propagation of vectors was purchased from Invitrogen. *P. pastoris* GS115 used for the expression of recombinant vector was obtained from Invitrogen. Yeast extract peptone dextrose (YPD), yeast extract peptone (YP), buffered glycerol-complex medium (BMGY), and buffered minimal methanol medium (BMM) were used to culture the methylotrophic yeast, *P. pastoris* and were prepared according to the manual of Easy select *Pichia* expression kit (Invitrogen).

2.3. Cloning of SLAC gene from Streptomyces coelicolor

SLAC encoding gene was amplified using the forward primer containing the *Eco*RI site and reverse primer containing the *Xba*I site (Table 1). PCR reaction was conducted with the following cycling conditions: Preheating 95 °C for 1 min, followed by 35 cycles of (95 °C for 40 s, 55 °C for 30 s, 72 °C for 2 min), and a final extension at 72 °C for 10 min. The resulting PCR product was double digested by *Eco*RI and *Xba*I and purified by GeneJet purification Kit (Thermofischer scientific). The digested PCR product was then ligated into digested pPICZαA vector and transformed into *E. coli* DH5α. Transformants were selected on low salt LB supplemented with 25 μg/ml zeocin.

2.4. Transformation of Pichia pastoris and screening of recombinant clones

Positive recombinant (*pPICZαA-SLAC*) was linearised using *Sac*I for transformation in *P. pastoris* GS115 through electroporation. Approximately 10 μg of linearized plasmid was added to yeast competent cells and pulsed at 1.5 kV, capacitance of 25 μF and 200 Ω of resistance. The cells were recovered with 1.0 M sorbitol and the mixture was transferred on YPD agar (g/l: yeast extract 10, peptone 20 and dextrose 20, agar 20) medium supplemented with 1.0 M sorbitol containing different concentrations of zeocin (100, 200, 500, 1000 and 1500 μg/ml). The plates were incubated at 30 °C until single distinct colonies appeared. Colony PCR was performed to identify positive transformants [8]. The positive clones were again transferred to fresh YPD agar plates (supplemented with 1.0 M sorbitol) with varying concentrations of zeocin (as described above). Small scale expression trials were performed to screen for the best producer of rSLAC. The transformants were first grown in YPD broth and then the biomass was pelleted (1500 x g, 10 min) and transferred to YP medium consisting of 0.5% (v/v) methanol to an OD_{600} of 1.0. The culture was cultivated at 30 °C, 250 rpm with 0.5% (v/v) methanol being added every 24 hours for 7 days. The culture supernatant was harvested by centrifugation at 4000 × *g* for 10 min at 4 °C and quantitative laccase assay was performed. The best clone was selected for further optimisation.

2.5. Optimisation of rSLAC production

Various parameters such as the different methanol concentration (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) and different media composition (YP and BMMY) were tested to determine the contribution of each parameter in overproduction of rSLAC. The cell biomass was generated in YPD/BMGY for YP/BMMY, respectively. Recombinant *P. pastoris* was inoculated in 50 ml YPD/BMGY medium in 250 ml conical flasks and was cultivated at 30 °C, 250 rpm until the OD_{600} reached 2.0-6.0. The cells were harvested during the log phase and added into the induction medium (YP/BMMY) to an OD_{600} of 1.0. The culture was incubated at 30 °C, 250 rpm with continuous addition of methanol at 24-h intervals to achieve concentrations ranging from 0.5% to 3.0%. Samples (100 μl) were withdrawn every day and cell biomass and laccase activity was determined. The effect of two non-repressing carbon sources (mannitol and sorbitol) in culture medium in combination with regular methanol induction was also studied. All experiments were performed in triplicate and results expressed as mean ± standard deviation.

2.6. Purification of recombinant SLAC

Cell free culture medium (YP) was collected by centrifugation (4000 × *g* for 30 min). The crude enzyme (supernatant) was concentrated by ammonium sulphate precipitation (30–80%) at 4 °C. The fractions were desalted by overnight dialysis (dialysis tubing; MW cut-off 14,000) and dialysed samples were incubated with $CuSO_4$ to restore enzymatic activity. The concentrated enzyme was then purified by fast protein liquid chromatography (FPLC) system [ÄKTApurifier 100, GE Healthcare, Bio-Sciences, Uppsala, Sweden] using HiTrap Capto Q column (GE Healthcare, Bio-Sciences, Uppsala, Sweden) and eluting the bound protein with a linear gradient of 0–1 M NaCl in Tris-HCl buffer (20 mM, pH 8.0) at 1.5 ml/min. The fractions with laccase activity were pooled and dialyzed against Tris–HCl buffer (20 mM, pH 8.0) and concentrated using a 10 kDa amicon ultra-15 centrifugal filter. The concentrated enzyme was loaded on a gel filtration chromatography column [Sephacryl S-300HR(26/60)] and eluted with 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl at a flow rate of 0.8 ml/min. The fractions having laccase activity were collected and their purity was determined using SDS-PAGE. The purified enzyme was used for enzyme characterisation.

2.7. Determination of molecular mass

Molecular mass of the active fraction was determined by SDS-PAGE by comparing the electrophoretic mobility of the enzyme to

that of the markers of known molecular weight. SDS-PAGE was carried out by using 12% resolving gel and 5% stacking gel, which was further stained by Coomassie brilliant blue G-250.

2.8. Laccase activity assay and protein determination

Laccase activity was determined at room temperature (22–25 °C) using ABTS, SGZ and 2,6-DMP as substrates, as described previously [22,23] Oxidation of ABTS (1 mM) was measured at 420 nm (ε = 36,000 M^{-1} cm^{-1}) in 20 mM acetate buffer (pH 4.0). The oxidation of SGZ (0.1 mM) was determined at 525 nm (ε = 65,000 M^{-1} cm^{-1}) in Tris-HCl buffer (pH 8.0). The oxidation of 2,6-DMP (2 mM) was determined at 466 nm (ε = 14,700 M^{-1} cm^{-1}) in Tris-HCl buffer (pH 8.0). The pH used depended on the optimum pH for the oxidation of the substrates used. The Lowry method was used to determine the protein content with bovine serum albumin as a standard [24].

2.9. Biochemical characterization of rSLAC

The optimum pH for the activity of rSLAC was determined by performing assays in different buffers [20 mM glycine-HCl buffer (pH 2.0, 3.0), 20 mM sodium acetate buffer (pH 4.0, 5.0), 20 mM Tris–HCl buffer (pH 6.0–8.0) and 20 mM glycine-NaOH buffer (pH 9.0)] at 80 °C. Optimum temperature was determined by conducting enzymatic assays at different temperatures (30 °C–90 °C) at the optimum pH for the substrate.

2.10. Enzyme kinetics and thermal deactivation of the rSLAC

Kinetic parameters of purified laccase were determined at 70 °C and 80 °C using different concentrations of ABTS (10–1000 μM), SGZ (5–100 μM) and 2,6-DMP (50–2000 μM). Assays were performed at each substrate's optimal pH (pH 4.0 for ABTS and pH 8.0 for SGZ and 2,6-DMP) and the K_m and k_{cat} values were determined. Activation energy (E_a) was calculated according to Ranjan et al. [25]. The energy of deactivation (E_d) of the enzyme was calculated from residual activity at different temperatures by incubating the enzyme solution in 20 mM sodium acetate buffer (pH 4.0) for ABTS and 20 mM Tris-HCl buffer (pH 8.0) for SGZ and 2,6-DMP at various temperatures (60–80 °C) in the absence of substrate. Aliquots were drawn at the desired intervals and laccase assays performed for calculating the residual activities. Inactivation rate constants (K_d) and energy of deactivation (E_d) were calculated as explained below:

Thermal denaturation is a reaction in which the rate of enzyme deactivation (dC/dt) follows first order kinetics in relation to the concentration of the active enzyme (C):

$$dC/dt = -k_d C \quad (1)$$

which can also be expressed as

$$\ln[C_t/C_0] = -k_d t \quad (2)$$

where C_o is the initial concentration of the enzyme and C_t is the concentration of the enzyme at time t. Since the residual enzyme activity (E) is directly proportional to the concentration of the active enzyme (C), thus,

$$E_t/E_0 = C_t/C_0$$

Now Eq. (2) can be written as:

$$\ln[E_t/E_0] = -k_d t$$

or

$$2.303\log[E_t/E_0] = -k_d t \quad (3)$$

K_d is the deactivation rate constant, which is calculated from the slope of the plots of $\log [E_t/E_0]$ vs. t. The half-life ($t_{1/2}$) of the enzyme is defined as the time required for the enzyme to lose half its initial activity. It can be expressed as follows:

$$t_{1/2} = 2.303\log 2/K_d \quad (4)$$

Energy required for deactivation (E_d) has been calculated by Arrhenius plot using the Arrhenius equation:

$$K_d = Ae^{(-E_d/RT)} \quad (5)$$

So that

$$\ln[K_d] = -E_d/RT + \ln A \quad (6)$$

where E_d represents energy of deactivation, R is the universal gas constant (8.314 J/K/mol) and T the absolute temperature. Deactivation energy (E_d) involved in the deactivation process is calculated from the slope of a linear plot of $\ln[K_d]$ vs T^{-1}. The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to a raise in the temperature by 10 °C. Temperature quotient (Q_{10}) was calculated by rearranging the equation of Dixon and Webb.

$$Q_{10} = \text{antilog}(E_a \times 10/RT^2) \quad (7)$$

Where E_a = activation energy, R is universal gas constant and T is absolute temperature.

2.11. Thermodynamic parameters of the rSLAC

Thermodynamics of irreversible inactivation of the rSLAC was determined by rearranging the Eyring's absolute rate equation derived from the transition state theory:

$$K_d = (K_b T/h)e^{(-\Delta H/RT)}e^{(-\Delta S/RT)}$$

ΔH (change in enthalpy of deactivation), ΔG (change in free energy of inactivation), and ΔS (change in entropy of inactivation) for irreversible inactivation were calculated as follows:

$$\Delta H = E_d - RT \quad (8)$$

$$\Delta G = -RT\ln(K_d h/K_b T) \quad (9)$$

$$\Delta S = (\Delta H - \Delta G)/T \quad (10)$$

where K_b is the Boltzmann's constant (R/N) = 1.38×10^{-23} J/K, T is the absolute temperature (K), h the Planck's constant = 6.626×10^{-34} J s, N is the Avogadro's number = 6.02×10^{23} mol^{-1}, R is the gas constant = 8.314 J/K/mol.

2.12. Decolourisation of dyes by rSLAC

The purified rSLAC was tested for the decolourisation of two synthetic dyes, Brilliant Blue G (Fig. S1A) (BBG, λ_{max} 630 nm) and Trypan Blue (Fig. S1B) (TB, λ_{max} 600 nm) in the absence of mediators. The reaction mixture (500 μl) consisted of 20 mM buffer (sodium acetate pH 4.0, Tris–HCl pH 6.0 and 8.0) containing dyes (50 mg/l, final concentration) and purified rSLAC (1 U/ml, activity in reaction mixture). The reaction was carried out at 37 °C with mild shaking (50 rpm) for 12 h. In order to rule out the event of abiotic decolourisation, a control reaction was also run in parallel where a reaction was set up with the denatured enzyme (boiled for 30 min) under identical conditions. The decolourisation of Brilliant Blue G and Trypan Blue dyes were spectrophotometrically determined as the relative decrease of the maximum absorbance wavelength of 630 nm and 600 nm, respectively. Decolourisation assays were performed in triplicate and percentage decolourisation was calculated according to [26] as shown below:

$$\%\text{Decolourisation} = [(I - F)]/I] * 100$$

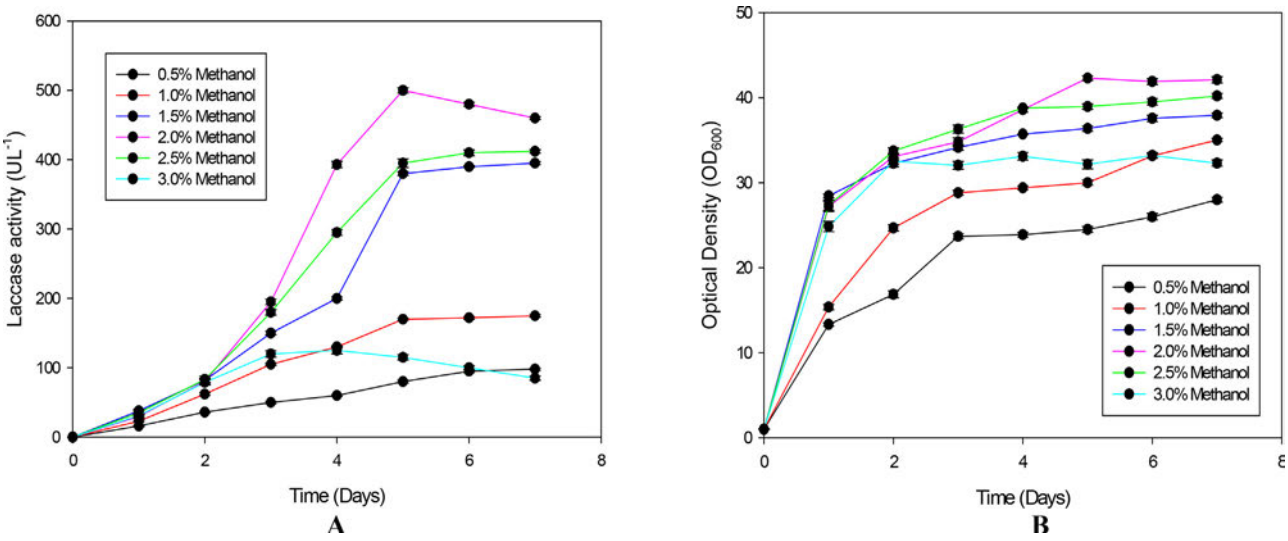

Fig. 1. Effect of methanol concentration (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) on (A) rSLAC activity and (B) cell biomass production of recombinant *P. pastoris* during growth in YP medium. All values are means ± standard deviation.

Where, I = initial absorbance and F = Absorbance after decolourisation

3. Results

3.1. Cloning and expression of SLAC in Pichia pastoris

The SLAC gene (ATCC SCO6712) of 1029 nucleotides that translates into a protein of 343 amino acids, was cloned in pPICZαA. The pPICZαA consisted of an AOX1 promoter for tight regulation, and methanol induced expression of the gene cloned under it. The recombinant plasmid *pPICZαA-SLAC* (Fig. S2) was confirmed by colony PCR and double digestion with *Eco*RI/*Xba*I (Fig. S3). The confirmed construct (*pPICZαA-SLAC*) was transformed into the competent *P. pastoris* GS115 cells. The transformants were tested for laccase expression via fermentation in small shake flasks by culturing in YP/BMMY medium under methanol inducible conditions. Among 150 clones screened, clone no.128 showed highest enzyme activity and was selected for further investigations. No cell-bound laccase activity was observed, thus showing efficient functioning of secretory signal sequence (α-factor). The transformant, which showed highest activity, was chosen for production of the rSLAC using YP medium. The produced rSLAC was then investigated for laccase activity using three laccase substrates (ABTS, SGZ and 2,6-DMP). All the substrates were successfully oxidised by the rSLAC.

3.2. Optimisation of rSLAC production in Pichia pastoris

Clone no. 128, harbouring *pPICZαA-SLAC* was cultured to optimise the expression of rSLAC under various conditions. YP medium was found to be the best medium for enzyme production. Thereafter, YP medium was used for further optimisation studies. Among different concentrations of methanol tested, 2% methanol was found to be the most effective concentration for enhancing rSLAC production; approximately 500 ± 10 U/l was produced (Fig. 1A). Preincubation of rSLAC at 80 °C led to ~7 fold increase in laccase activity (using ABTS as a substrate).

3.3. Purification of rSLAC and determination of molecular mass

The rSLAC was purified to homogeneity and the purity was confirmed by SDS-PAGE. Purification was performed in three steps viz. ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The extracellular rSLAC was purified to 60.35 fold with a specific activity of 8916.66 U/mg (Table 2). After gel filtration on Sephacryl S-300HR(26/60), the active fraction of SLAC (Fig. S4) was ran on SDS-PAGE to determine the molecular mass. The purified SLAC appeared as a single band corresponding to a molecular mass of ~38 kDa on SDS-PAGE (Fig. 2) and was in agreement with the predicted molecular weight derived from the gene sequence (1029 bp).

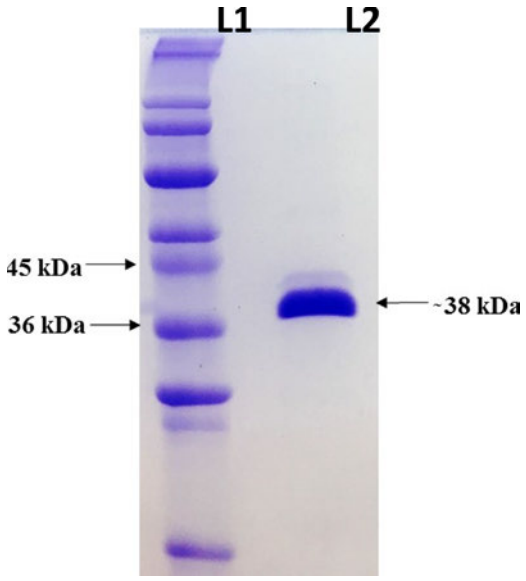

Fig. 2. SDS-PAGE analysis of the purified rSLAC: L1 protein marker; L2 purified rSLAC.

3.4. Purification of rSLAC

The rSLAC was purified to homogeneity and the purity was confirmed by SDS-PAGE. The band corresponded to ~38 kDa on SDS PAGE (Fig. 2) and was in agreement with the predicted molecular weight derived from the gene sequence (1029 bp). Purification was performed in three steps viz. ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography. The extracellular rSLAC was purified to 60.35 fold with a specific activity of 8916.66 U/mg (Table 2).

Table 2
Purification summary of rSLAC produced in *Pichia pastoris*.

Purification steps	Total activity (U/ml)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold
Crude enzyme	3.8	0.0257	147.74	1
$(NH_4)_2SO_4$	21.6	0.0431	501.28	3.39
Anion exchange	16.4	0.0030	5559.32	37.62
Gel filtration	9.63	0.0011	8916.66	60.35

Table 3
Kinetic parameters of recombinant bacterial laccases.

Recombinant bacterial laccases	ABTS		SGZ		2, 6-DMP		References
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	
rSLAC (*P. pastoris*)	142.85	60.6	10.86	25.36	54.55	27.84	This study
SLAC (*E.coli*)	5893	9.49	ND	ND	5088	8.22	Prins et al. [23]
rcotA (*P. pastoris*)	146.4	14.4	12.7	6.9	ND	ND	Wang et al. [40]
rcotA (*P. pastoris*)	162	15	24	7.6	166	0.87	Fan et al. [9]

ND – not determined.

Table 4
Thermodynamic parameters of rSLAC measured during thermal deactivation at various temperatures.

Temp. (K)	K_d (h^{-1})	$T_{1/2}$ (h)	ΔH (KJ/mol)	ΔG (KJ/mol)	ΔS (J/mol K)
333.15	0.0116	60	114.505	94.279	60.71
343.15	0.0217	32	114.422	95.392	55.45
353.15	0.0693	10	114.339	94.841	55.21

3.5. Biochemical characterisation of rSLAC

The pH optima for oxidising ABTS, SGZ and 2,6-DMP was found to be 4.0, 8.0 and 8.0, respectively (Fig. 3A). The highest activity was observed at 80 °C with all the substrates (Fig. 3B).

3.6. Kinetic and thermodynamic characterisation of rSLAC

The kinetic constants, K_m values for ABTS, SGZ and 2,6-DMP were 142.85 μM, 10 μM and 54.55 μM with corresponding k_{cat} values of 60.6 s^{-1}, 25.36 s^{-1} and 27.84 s^{-1}, respectively (Table 3). The activation energy (E_a) for ABTS oxidation by rSLAC was calculated and found to be 38.5 kJ/mol. The deactivation constant (K_d) of rSLAC was calculated from the plot of the $\ln(E_t/E_0)$ versus time (Fig. 4A). The K_d value was substituted in Eq. (4) to calculate $t_{1/2}$ of the rSLAC. The $t_{1/2}$ values of the rSLAC at 60 °C, 70 °C, 80 °C were found to be 60 h, 32 h and 10 h respectively. Arrhenius plot of the deactivation constant for rSLAC (Fig. 4B) at different temperatures was plotted to calculate the deactivation energy (E_d), which was found to be 117.275 kJ/mol. First order kinetics was applied on thermal inactivation data to determine the thermodynamic parameters of thermal inactivation of rSLAC. The values ΔG, ΔH and ΔS for thermal inactivation of rSLAC were positive (Table 4).

3.7. Application of rSLAC in dye decolourisation

Maximum dye decolourisation was achieved within 6 h (Fig. 5A) and the dyes were better decolourised in acidic than alkaline pH (Fig. 5B). Approximately 92.2% of BBG and 91.7% of TB were decolourised (pH 4) by the rSLAC without the aid of a mediator compared to 86.2% of BBG and 46.7% TB at pH 8.0 (Fig. 5B). In the control reaction, no decolourisation was observed indicating the catalytic role of rSLAC in dye decolourisation.

4. Discussion

Low production yield is a major hurdle for application of bacterial laccases at industrial level. Improving production of laccases can therefore enhance their application potential. Laccases from actinobacteria are widely distributed among different *Streptomyces* spp., notably *S. coelicolor* [15,27–32]. Biotechnological interest in the small laccase produced by *S. coelicolor* has increased in recent years due to its stability under high temperatures and activity within a wide range of pH [7,33]. In the present study, we have cloned the *SLAC* gene into the pPICZαA vector using an AOX1 promoter and α factor as a secretion signal. The AOX1 promoter allowed for a high expression level of SLAC in *P. pastoris* by methanol induction. Furthermore, in order to enhance the yield of protein expression, medium composition and methanol concentration were optimised. Of the two media tested, YP medium was found to be more effective in enhancing the rSLAC production. Similar results have been obtained for other recombinant enzymes using *P. pastoris* as an expression host [8]. Since methanol is a well-known inducer in AOX1-based *Pichia* cultivations [34] as well as an important factor in optimisation of recombinant protein production [35], we therefore tested different percentages of methanol to enhance the rSLAC production. Induction by 2% methanol was found to be most effective. Reduction in production level after 2% methanol concentration could be due to oxygen depletion which can in turn negatively affect protein expression [36].

Maximum production of rSLAC (500 ± 10 U/l) was achieved after 5 days of fermentation, which is significantly higher than other bacterial laccase expressed in *P. pastoris* [17]. However, bacterial CotA laccase expressed in *P. pastoris* achieved maximum production in 12 days, which is possibly due to the use of a different strain of *P. pastoris* (SMD1168H) [9]. We also studied the laccase activity after preincubation of enzyme at higher temperatures. It is worth noting that the laccase activity increased up to~7 fold after 30 min incubation at 80 °C. Similarly, preincubation also enhanced laccase activity in CotA [37] and other thermostable laccases [38]. Pre-incubation possibly reduces the time for enzyme to reach the activation energy, which increases the rate of reaction. Pre-incubation could also possibly increase substrate accessibility to active sites through reduction in enzyme aggregation.

The rSLAC secreted into the culture medium was purified to homogeneity and visualised as a single band of ~38 kDa on SDS-PAGE. The size of rSLAC is similar to those of EpoA and SCLAC [29,30]. Thereafter, kinetic and thermodynamic parameters were evaluated. The value of K_m, V_{max} and k_{cat} were calculated for three substrates (ABTS, SGZ and 2,6-DMP) and compared to the other recombinant bacterial laccases (Table 3). In the present kinetic study, we observed that K_m values for all substrates (ABTS, SGZ and 2,6-DMP) were lower than other laccases [9,14,39,40], which indicates that rSLAC has a higher affinity towards the investigated substrates. We also determined the catalytic efficiency of rSLAC

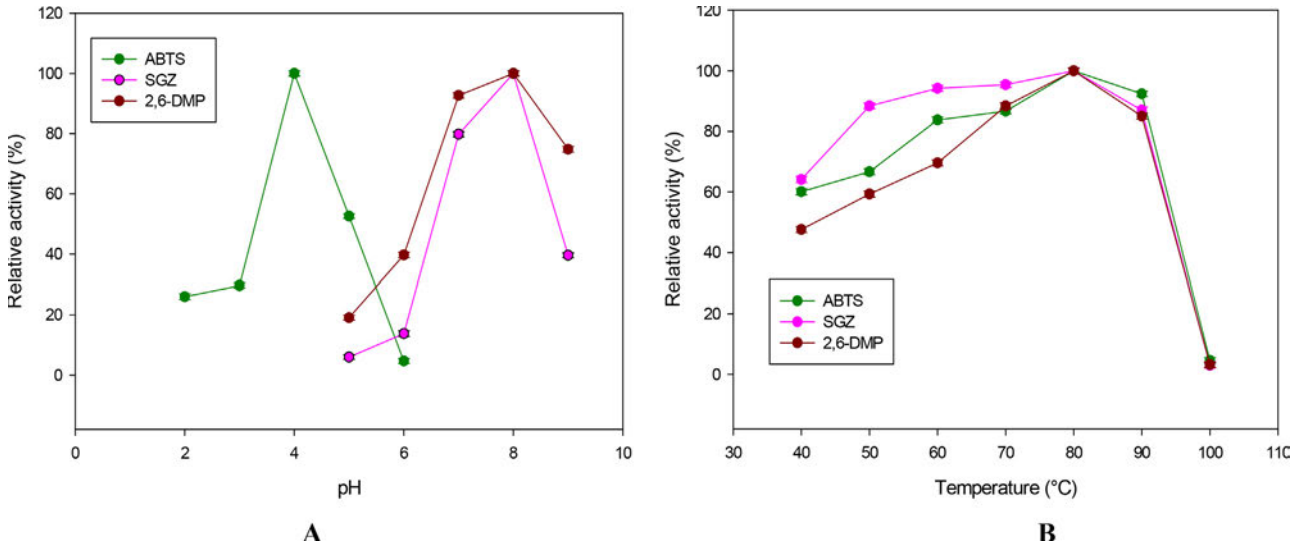

Fig. 3. Effect of pH (A) and temperature (B) on rSLAC activity towards ABTS (1 mM), SGZ (0.1 mM), and 2, 6-DMP (2 mM). All results are presented as means ± standard deviation.

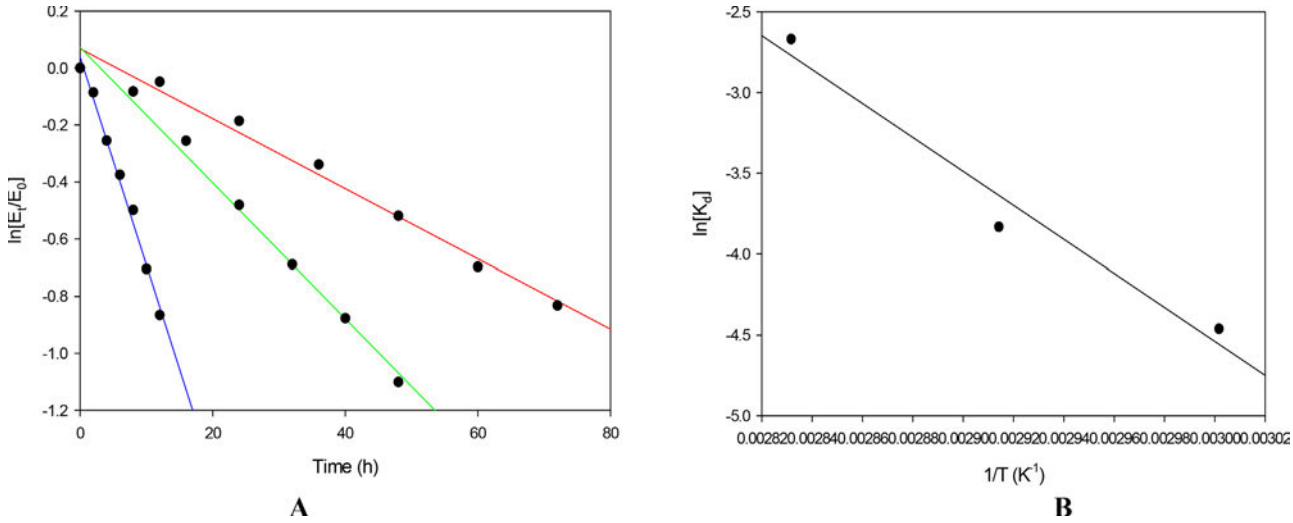

Fig. 4. A. Plot of $\ln[E_t/E_0]$ versus time (min) for the calculation of deactivation constant (K_d) and $t_{1/2}$ of rSLAC at different temperatures [60 °C (red line), 70 °C (green line), 80 °C (blue line)]. B. Arrhenius plot of rSLAC for the calculation of deactivation energy (E_d). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

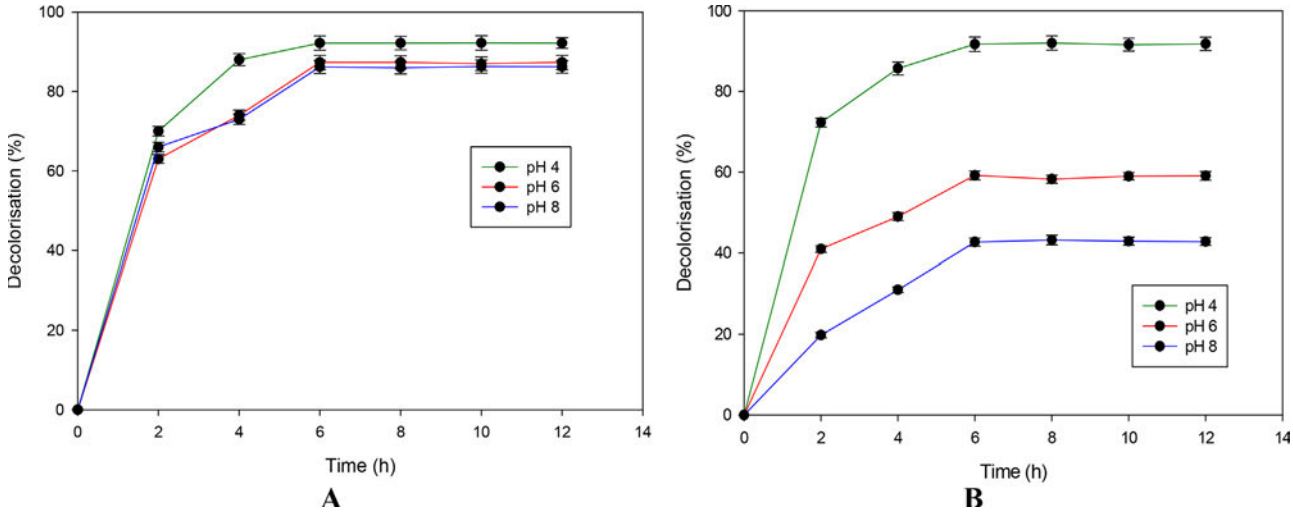

Fig. 5. Effect of time and pH on dye decolourisation by purified rSLAC: A Brilliant Blue G and B Trypan blue. All values are means ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which was found to be higher than other known bacterial laccases [9,17,40]. This indicates that a small amount of rSLAC is potentially more potent in applications [41]. This is supported by findings from the present study which showed that only 1 U of rSLAC was efficient in decolourising more than 90% of dyes without the assistance of a mediator. However, 9 U of rCotA was required to decolourise 95% indigo carmine dye in the presence of mediator [40].

Thermostability of an enzyme molecule is defined as its ability to resist thermal inactivation; the higher the value of Ed, the more thermostable is the enzyme. Moreover, thermodynamic parameters also provide a detailed picture of numerous chemical and biological reactions [42]. Thermal inactivation of enzyme consists of two steps: Native (N) ↔ Unfolded (U) → Inactive (I), where N stands for native enzyme, U for unfolded inactive enzyme and I for inactivated enzyme [43]. When enzyme is subjected to high temperatures, unfolding of native enzymes takes place resulting in the formation of unstable intermediate (U) which could either remain in this state till the input energy is less than the inactivation energy [44] or reversibly refold upon cooling [45]. However, prolonged exposure to heat can lead to crossing of the inactivation barrier, where unfolding occurs and the enzyme is permanently inactivated [45]. The higher the value of deactivation energy (E_d), the longer it will be in its native conformation, after being subjected to high temperatures. In the present study, we also found that rSLAC has significantly higher E_d value of 117.275 kJ/mol, which would allow rSLAC to withstand changes to its native conformation when exposed to high temperatures. In addition, we have also calculated other thermodynamic parameters, such as ΔG, ΔH, and ΔS for the rSLAC. Viability of any chemical reaction is mainly determined by the measurement of change in Gibbs free energy (ΔG), specifically, the transformation of E–S complex into products. The lower the ΔG, the more viable is the reaction. Strikingly, we found that rSLAC has lower ΔG compared to that reported for a fungal laccase [46]. Also, the positive free energy change (ΔG) for the rSLAC indicates the non-spontaneous nature of the thermal denaturation of recombinant enzyme. Besides Gibbs free energy (ΔG), the enthalpy change (ΔH) indicates the energy required for thermal denaturation of protein. The larger the ΔH value, the higher the energy required to break stabilising bonds in the thermal inactivation of the enzyme. Here, we found that rSLAC has higher ΔH value in comparison to the other fungal laccase previously studied [46]. Enzyme denaturation is also accompanied by increase in the disorder of the enzyme structure, measured in terms of entropy change (ΔS), which decreases with increasing enzyme stability. To the best of our knowledge, this is the first report on evaluation of thermodynamic parameters for any known bacterial laccases.

The purified rSLAC was found to be efficient in decolourising two synthetic dyes belonging to two different classes: triphenylmethane dye (Brilliant Blue G) and a di-azo dye (Trypan Blue), without the assistance of a mediator. We observed more than 90% dye decolourisation using rSLAC. However, in earlier reports, SLAC decolourised only 8% of an Indigo dye without the assistance of a mediator [7,33]. Among different pH (4, 6 and 8) tested we did not observe any significant effect of pH on decolourisation of Brilliant Blue G dye. This indicates that rSLAC remains efficient in acidic to alkaline range of pH. However, there was decrease in decolourisation of Trypan Blue (azo dye) in alkaline pH. Similarly, alkaline pH had negative effect on azo dye decolourisation by other laccases [47,48]. It is worth mentioning the recalcitrant nature of the azo group of dyes and the resistance to laccase-catalysed oxidation increases with increase in the number of azo groups [41]. Usually, decolourisation of azo dyes requires redox mediators [41,49–51], however, rSLAC was able to decolorise TB (azo dye) without the aid of a redox mediator [52], which is consistent with findings for CotA laccase [53]. This suggests that bacterial laccases have potential for decolourising recalcitrant dyes used in textile industries.

5. Conclusion

SLAC from *S. coelicolor* was functionally expressed and secreted in *P. pastoris*. Optimisation of production medium and methanol concentration enhanced rSLAC production. The rSLAC showed good decolourisation potential towards Brilliant Blue G and Trypan Blue. Extracellular expression of SLAC in *P. pastoris* opens up avenues for its wide application in various industries.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijbiomac.2017.11.169.

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