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PRODUCTION AND MOLECULAR CHARACTERIZATION OF PEROXIDASES FROM NOVEL LIGNINOLYTIC PROTEOBACTERIA AND BACILLUS STRAINS

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PRODUCTION AND MOLECULAR CHARACTERIZATION OF PEROXIDASES FROM NOVEL LIGNINOLYTIC PROTEOBACTERIA AND BACILLUS STRAINS

BY

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2018

DECLARATION

I, the undersigned, declare that this thesis entitled *"Production and molecular characterization of peroxidases from novel ligninolytic proteobacteria and bacillus strains"* submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Biochemistry in the Faculty of Science and Agriculture is my original work and that the work has not been submitted to any other University in partial or entirely for the award of any degree or examination purposes.

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…… ……

Prof. L. V. Mabinya Date

Supervisor

…… ……

Prof. U. U. Nwodo Date

Co-supervisor and Head of Department

DEDICATION

This thesis is dedicated to God Almighty, the giver of life; and in loving memory of my late Father, Mr. Johnson Akin Falade, who passed on in the course of this study.

"...it is not of him that willeth, nor of him that runneth, but of God that sheweth mercy"

Rom. 9:16 (KJV)

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LIST OF SUBMITTED MANUSCRIPTS

i. Versatile peroxidase functionality: elimination of endocrine disrupting chemicals in wastewater.

ii. Peroxidase produced by ligninolytic Bacillus species isolated from marsh and grassland decolourized anthraquinone and azo dyes.

iii. Optimized peroxidase production and detection of catalase-peroxidase gene (*KatG*) in a Bacillus species isolated from Hogsback forest reserve, South Africa.

iv. Optimization of process parameters for exoperoxidase production by *Ensifer adhaerens* NWODO-2 and PCR detection of catalase-peroxidase gene (*KatG*).

v. Peroxidase production by a novel ligninolytic proteobacteria strain: *Raoultella ornithinolytica* OKOH-1.

vi. Biochemical and molecular characterization of *Raoultella ornithinolytica* peroxidase with biotechnological potentials in dye decolourization and development of cosmetic agent.

LIST OF ACRONYMS

2, 4-DCP:	2, 4-Dichlorophenol
ABTS:	2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
AC:	Ammonium Chloride
AEMREG:	Applied and Environmental Microbiology Research Group
AN:	Ammonium Nitrate
ANOVA:	Analysis of Variance
APEs:	Alkylphenol Ethoxylates
APs:	Alkylphenols
AS:	Ammonium Sulphate
AZB:	Azure B
B[a]P:	Benzo [a] Pyrene
BBD:	Box-Behnken Design
BGL:	β-glucosidase
BLAST:	Basic Local Alignment Search Tools
BPA:	Bisphenol A
BSA:	Bovine Serum Albumin
CCD:	Central Composite Design
CcP:	Cytochrome c Peroxidase
CLEA:	Cross Linked Enzyme Aggregates
CR:	Congo Red
DDT:	Dichlorodiphenyltrichloroethane
DiHCcP:	Di-heme Cytochrome c Peroxidase
DNA:	Deoxyribonucleic Acid
DOPA:	Dihydroxyphenylalanine
DyP:	Dye decolourizing Peroxidase
EDC:	Endocrine Disrupting Chemicals
EDTA:	Ethylenediaminetetraacetic acid
EMR:	Enzymatic Membrane Reactor
FPase:	Filter Paper Cellulase
GA:	Guaiacol

GMRDC:	Govan Mbeki Research and Development Centre
HPI:	Hydroperoxidase I
HRP:	Horseradish Peroxidase
KatG:	Catalase-peroxidase
KL:	Kraft Lignin
KTBA:	α-keto-γ-thiomethylbutyric acid
LiP:	Lignin Peroxidase
LMEs:	Lignin Modifying Enzymes
LRET:	Long Range Electron Transfer
MEGA:	Molecular Evolutionary Genetics Analysis
MCO:	Multicopper Oxidase
MnP:	Manganese Peroxidase
NCBI:	National Centre for Biotechnology Information
NRF:	National Research Foundation
OD:	Optical Density
PAHs:	Polycyclic Aromatic Hydrocarbons
PAHs:	Polycyclic Aromatic Hydrocarbons
PCBs:	Polychlorinated biphenyls
PCP:	Pentachlorophenol
PCR:	Polymerase Chain Reaction
PhACs:	Pharmaceutically Active Compounds
PPCPs:	Pharmaceutical and Personal Care Products
RB4:	Reactive Blue 4
RBBR:	Remazol Brilliant Blue R
rDNA:	Ribosomal Deoxyribonucleic Acid
rRNA:	Ribosomal Ribonucleic Acid
RSM:	Response Surface Methodology
SAMRC:	South African Medical Research Council
SMF:	Submerged Fermentation
SNP:	Single Nucleotide Polymorphism
SSF:	Solid State Fermentation

STD:	Standard Deviation
TSS:	Two-Stage System
UniProtKB:	UniProt Knowledgebase
UV:	Ultra Violet
VA:	Veratryl Alcohol
VP:	Versatile Peroxidase
YEO:	Yeast Extract Only

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GENERAL ABSTRACT

Inadequate quantity is a major impediment to the industrial application of peroxidase and other industrial enzymes. Consequently, efforts are geared towards increasing peroxidase production by searching for new microbes with enhanced production capacity. In this study, three novel ligninolytic bacteria: *Raoultella ornithinolytica* OKOH-1 (KX640917), *Ensifer adhaerens* NWODO-2 (KX640918) and *Bacillus* sp. FALADE-1 (KX640922) were optimized for peroxidase production and their peroxidases characterized using molecular and biochemical approaches. Molecular analysis confirmed the presence of peroxidase genes in the three bacteria. BLAST result and phylogenetic analysis of the deduced amino acid sequences suggested that *Raoultella ornithinolytica* OKOH-1 peroxidase (RaoPrx) belongs to a DyP-type peroxidase family while peroxidases from *Ensifer adhaerens* NWODO-2 and *Bacillus* sp. FALADE-1 are catalase-peroxidases. The peroxidase genes are available in the GenBank with MF370527, MF374336 and MF407314 as respective accession numbers. Upon optimization, *Raoultella ornithinolytica* OKOH-1 exhibited the highest peroxidase production at pH 5, 35 ^{o}C and 150 rpm. Biochemical characterization showed that RaoPrx had a wide substrate specificity as it was able to oxidize all the tested substrates in this study (ABTS, veratryl alcohol, guaiacol and pyrogallol), except 2, 6-Dimethoxyphenol. However, highest activity by the enzyme was recorded with pyrogallol as substrate. The enzyme had an optimum activity at pH 6 and 50 ^{o}C and was very stable at high temperatures (50 ^{o}C – 70 ^{o}C). Its pH stability was over a pH range of 5.0 – 7.0. Moreover, RaoPrx activity was significantly enhanced by Ag^{+}, $Cu^{2+,}$ $Zn^{2+}$and Fe^{2+} while $Ca^{2+,}$ $Mg^{2+,}$ $Ba^{2+,}$ Al^{3+}, Co^{2+}, NaN_3 and EDTA inhibited the activity of the enzyme. Nevertheless, RaoPrx exhibited a remarkable dye-decolourizing activity on congo red and melanin, indicating the biotechnological potential of the enzyme in dye decolourization and development of cosmetic agent. Generally, the results from this study suggest that ligninolytic bacteria hold a great potential for enhanced peroxidase production that could meet the increasing industrial demand.

***Keywords*:** Catalase-peroxidase, dyp-type peroxidase, enzyme kinetics, enzyme production, enzyme technology, lignin modifying enzymes, lignin degradation, peroxidase gene.

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Background to the study

The recalcitrance of lignin to degradation constitutes an undesirable barrier to the efficient and optimum utilization of the abundant lignocellulosic resources. Thus, effective degradation of lignin is of prime importance to the industrial sectors utilizing lignocellulose as raw materials for various value-added products including ethanol. This has therefore necessitated the exploration of different physicochemical pretreatment methods such as steam explosion, ammonia fibre explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, organosolvation etc. However, most of these techniques are characterized by demerits such as high cost, high energy input, corrosion, release of compounds that may inhibit fermentation and material loss (Chaturvedi and Verma, 2013; Huang *et al.,* 2013). Hence, the imperativeness of developing an effective pretreatment method with less limitations. Biological methods, involving the use of microorganisms have been advocated as cheaper and ecofriendly alternatives (Falade *et al.,* 2017).

Ligninolytic activities of fungi have been extensively studied. However, commercialization of the biocatalytic process of lignin degradation by fungi still remains a challenge, perhaps, due to difficulty in the optimization of fungal enzyme production through genetic engineering (Bugg *et al.,* 2011). Thus, the search for novel ligninolytic bacteria has continued to increase, partly, owing to the maneuverability of the bacterial genome. As well, the emerging role of bacteria in lignin degradation has been reported (Bugg *et al.,* 2011).

Proteobacteria and Bacilli are part of the few classes of bacteria that have shown potential for lignin degradation (Ahmad *et al.,* 2010; Bandounas *et al.,* 2011; Chang *et al.,* 2014; Bao *et al.,* 2015). Proteobacteria are gram-negative bacteria and constitute one of the largest group of the prokaryotes (Zinder, 1998; Gupta, 2000). This group of bacteria are also known as "purple bacteria" (Olsen *et al.,* 1994), a nomenclature that has been regarded as inappropriate as purple colouration is restricted to only a small number of organisms in this group. Based on phylogenetic analysis (16S and 23S rRNA), proteobacteria is divided into five sub-classes: α (alpha), β (beta), γ (gamma), δ (delta) and ε (epsilon) proteobacteria (Woese *et al.,* 1984 a, b; 1985; Woese, 1987).

Proteobacteria includes a vast number of known human, animal and plant pathogens (Collier *et al.,* 1998). Apart from the biological significance of proteobacteria in pathogenesis, a number of bacteria belonging to α-proteobacteria (*Novosphingobium* sp. strain MBES04 and *Rhizobium* sp. strain YS-1r), β-proteobacteria (*Burkholderia* sp. strain LIG30 and *Cupriavidus basilensis* B-8)

and γ-proteobacteria (*Halomonas* sp. strain KO116, *Klebsiella* sp. strain BRL6-2 and *Raoultella ornithinolytica* strain S12) sub-classes have shown lignin degrading activities (Shi *et al.,* 2013; Woo *et al.,* 2014; Bao *et al.,* 2015; O'Dell *et al.,* 2015; Ohta *et al.,* 2015; Prabhakaran *et al.,* 2015; Kameshwar and Qin, 2016), hence, their industrial significance in the valorization of lignocellulosic biomass to value-added products.

Proteobacteria have also shown potential for production of products of economic importance such as enzymes and chemicals. Novel enzymes including α-galactosidase, β-galactosidase, phosphatase, α-amylase, protease and β-glucanase have recently been discovered in proteobacteria (Vester *et al.,* 2014). Production of laccase and lipase by proteobacteria has also been reported (Kalme *et al.,* 2009; Ghazali and Abdul-Hamid, 2015; Neifar *et al.,* 2016).

Furthermore, Raoultella species have been employed for production of pullulanase, a debranching enzyme hydrolyzing pullulan and branched polysaccharides (Hii *et al.,* 2012); and production of biomolecules such as polysaccharide-protein complex and tri-peptide complex (Fiolka *et al.,* 2013; 2015). Specifically, *Raoutella ornithinolytica* B6, produced 2,3-Butanediol (2,3 BD) as an alternative to the petroleum-based 2,3 BD production (Kim *et al.,* 2016, 2017). It is therefore evident that Raoutella species and other proteobacteria hold great biotechnological potentials.

Bacilli, is another class of bacteria with emerging ligninolytic activities (Chang *et al.,* 2014) and enormous industrial applicability. Bacillus species are referred to as the "major workhorse industrial microorganisms" (Schallmey *et al.,* 2004). Bacillus species have received attention as an industrial organism, partly due, to their high growth rate, consequently short fermentation cycle times. As well, they possess the ability to secrete extracellular proteins and are generally regarded as being safe (Schallmey *et al.,* 2004). Bacillus species are important industrial enzyme producers as they are capable of producing large quantities of enzyme. Organisms belonging to the genus Bacillus have shown the dexterity to synthesize extracellular enzymes including protease, amylase and lipase (Sevinc and Demirkan, 2011; Barros *et al.,* 2013; Pant *et al.,* 2015). Likewise, some Bacillus species have been employed for the production of cellulolytic and pectinolytic enzymes (Soares *et al.,* 2001; Dias *et al.,* 2014; Padilha *et al.,* 2015).

The emerging role of Proteobacteria and Bacillus species in lignin degradation, a secondary metabolic process, is notably accompanied by the production of extracellular lignin modifying

enzymes (LMEs) such as laccases and predominantly heme peroxidases (lignin peroxidase, manganese peroxidase, versatile peroxidase and dye-decolourizing peroxidase).

Besides the ligninolytic activities of peroxidases, they have also shown a great potential for industrial application in the development of skin-lightening agents (Draelos, 2015; Falade *et al.*, 2017), removal of endocrine disrupting chemicals (EDCs) in wastewater (Taboada-Puig *et al.*, 2015) and degradation of synthetic dyes (Kalyani *et al.,* 2011). Nevertheless, the industrial application potentials of peroxidases are hampered by high production costs and inadequate quantity of enzyme produced (Ferrer *et al.,* 1991; Torres *et al.,* 2003). This has therefore necessitated the search for novel ligninolytic organisms with enhanced peroxidase production capacity in the freshwater and terrestrial milieux of the Raymond Mhlaba Municipality, Eastern Cape, South Africa.

1.2. Statement of the research problem

Lignin constitutes a barrier to biochemical hydrolysis of cellulose and hemicellulose, making biomass inherently resistant to treatment (Huang *et al.,* 2013) and this has been a major bottleneck in the industrial application of lignocellulosic biomass. Although several physical and chemical methods of pretreatment have been developed to address this challenge, these methods are expensive; require significant energy inputs, release compounds that inhibit fermentation, cause corrosion and probably lead to loss of materials (Huang *et al.,* 2013). These demerits inform the need to explore alternative means of delignification involving the use of ligninolytic microbes or immobilized microbial sub-molecules including enzymes.

The application of fungal enzymes in the delignification of lignocellulosic biomass has been explored but yet to be commercialized (Bugg *et al.,* 2011). Perhaps, the insufficiency in the maneuverability of the fungal genome for optimum extracellular enzyme yield, as a function of production cost to commercial value quotient may have constituted an important factor impeding commercialization of the process (Ausec *et al.,* 2011; Bugg *et al.,* 2011). Thus, the need for the exploitation of bacteria for production of ligninolytic enzymes including peroxidases is imperative.

Besides, the interest in the biotechnological applications of peroxidases has continued to increase partly, due to their high redox potential for oxidation of recalcitrant compounds including phenolic and non-phenolic aromatic compounds.

Given the promising industrial application potentials of peroxidases, their production in large quantity is of utmost importance as enhanced enzyme production is one of the significant requirements for an effective bio-catalytic process. As well the search for novel peroxidases with excellent industrial physicochemical characteristics is imperative.

1.3. Hypothesis

This study was premised on the hypothesis that bacteria strains from freshwater and terrestrial environments of the Raymond Mhlaba Municipality, Eastern Cape, South Africa are not capable of producing peroxidases with potential industrial applications.

1.4. Aim and objectives

1.4.1. Research aim

This study aimed at exploring the freshwater and terrestrial environments of the Raymond Mhlaba Municipality, Eastern Cape, South Africa for bacteria strains with enhanced peroxidase production capacity.

1.4.2. Research objectives

The specific objectives are to:

i. collect samples from selected freshwater and terrestrial environments for the isolation of ligninolytic bacteria species;
ii. screen isolates for ligninolytic and peroxidase production potentials;
iii. identify the positive isolates using 16S rDNA sequence analysis;
iv. optimize the culture conditions for peroxidase production;
v. characterize the genes encoding peroxidase production; and
vi. determine the biochemical characteristics and evaluate the peroxidase from the most efficient producer for dye decolourization and melanin oxidation.

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CHAPTER TWO

LITERATURE REVIEW

(Part published in Microbiology Open as lignin peroxidase functionalities and prospective applications)

2.1. Introduction

The espousal for the utilization and perhaps, the utilization of lignocellulosic biomass for the production of value-added products is on the increase world over, partly, due to the abundance and renewable nature of lignocellulosic biomass. Woody and non-woody plants possess lignocellulose as major structural components and two carbohydrate polymers viz. cellulose (~30-50%) and hemicelluloses (~15-30%) as well as some non-carbohydrate aromatic polymers (~15-30%) constitute lignocellulose (Foyle *et al.*, 2007; Harris and Debolt, 2010; Menon and Rao, 2012). In woody or herbaceous plants, the lignocellulose constituent varies in accordance with the species and in tandem with the biotic and abiotic stressor factors including environmental distress syndromes.

Several categorizations have applied to lignocellulosic biomass including; waste biomass, virgin biomass, and energy crops. Waste biomass are thought to be low value by-products largely generated from industrialized forestry activities (sawdust, wood waste, pulp mill waste), agricultural practices (corn stover/cob, sugarcane bagasse, wheat straw, rice husks, animal droppings) and municipal solid waste. On the other hand, terrestrial plants are classified as virgin biomass while energy crops include those generating large amount of lignocellulosic biomass as feedstock for second generation biofuel production.

Increased generation of lignocellulosic wastes from both the industrial and agricultural sectors have continued to pose environmental challenge globally due to, in part, poor waste management. However, the prospect of the valorization of lignocellulosic wastes for value-added products shall suffice as effective waste management strategies. Nonetheless, this proposition is at the moment underexplored.

Besides, valorization of lignocellulosic wastes avails the right set of circumstance for the harnessing of value added products from the compositional structures of the lignocellulosic biomass. The valorised may be, in effect, the products of interest for end users otherwise they may serve as raw materials for the production of commercially viable products. On the converse, microbial activities on lignocellulosic biomass during valorization process may also generate industrially important products including enzymes, ethanol, organic acids, microbial polysaccharides and vitamins. Irrespective of any delineated path that may lead to products of

interest, lignin recalcitrance to degradation has remained a major bottleneck to various industrial operations.

2.2. Lignin degradation and ligninolytic organisms

Lignin has constituted a major hindrance in the valorization of lignocellulosic biomass into value-added products due to its recalcitrance to degradation. Besides the conferral of hydrolytic stability and structural rigidity to plant's cell walls, lignin traps and renders unavailable the saccharides constituting the mono-, di-, oligo- and poly-meric units of cellulose necessary for fermentation. Lignin is imperative for the survival of plants and its recalcitrance to degradation has been attributed to its cross linkages with polysaccharides (cellulose and hemicellulose) via ester and ether linkages and as well, its molecular architecture, in which various non-phenolic phenylpropanoid units produce a complicated three-dimensional network joined by an array of ether and carbon-carbon bonds (Ruiz-Dueñas and Martinez, 2009).

In a bid to address the challenge of lignin recalcitrance to degradation, several physicochemical pre-treatment technologies have been developed to disrupt the non-cellulosic matrix and render cellulose and hemicellulose more accessible for enzymatic hydrolysis (Mosier *et al.,* 2005). Some of these methods include steam explosion, ammonia fiber explosion, acid hydrolysis, alkaline hydrolysis, ozonolysis, organosolvation and oxidative delignification (Chaturvedi and Verma, 2013). These pretreatment technologies are generally expensive, require high energy inputs, generate compounds inhibitory to fermentation, release toxic chemicals which leads to corrosion problems and may also lead to material loss (Chaturvedi and Verma, 2013; Huang *et al.,* 2013). Nonetheless, the biological method of delignification may serve as an alternative pretreatment process as it is saddled with fewer limitations (Kuhar *et al.,* 2008; Huang *et al.,* 2013). Biological pretreatment involves the use of microorganisms or immobilized microbial sub-molecules such as enzymes. The method may be thought of as cheap and environmental-friendly. However, it is not without demerits which include utilization of, part of, the fermentable sugars as carbon source, thus consequently lowering product yield (Wan and Li, 2011; Potumarthi *et al.,* 2013).

Lignin degradation has been extensively studied in wood-rotting organisms, especially white-rot basidiomycetes (Hatakka, 1994; Leonowicz *et al.,* 1999; Martinez *et al.,* 2004; Wan and Li, 2012), and most of these studies established white-rot fungi as the most effective "delignifyer". Besides

the white-rot and brown-rot fungi, some bacteria have also been reported to possess ligninolytic abilities with the potential of producing ligninases. This group of bacteria has been classified as; actinomycetes, α-proteobacteria, ᵞ-proteobacteria (Bugg *et al.,* 2011; Paliwal *et al.,* 2012). Likewise, members of the Bacillus genus with ligninolytic abilities have recently been reported (Bandounas *et al*., 2011; Chang *et al.,* 2014; Zhu *et al.,* 2017). However, the mechanisms of lignin degradation differ by bacteria, while some utilize extracellular enzymes such as dye-decolourizing peroxidase, laccase and manganese superoxide dismutase to modify lignin (Majumdar *et al.,* 2014; Rashid *et al.,* 2015), others employ the gentisate, benzoic acid and β-ketoadipate degradation pathways (Ahmad *et al.,* 2010; Zhu *et al.,* 2017). A list of some reported ligninolytic bacteria and their classes is given in Table 2.1.

Table 2.1. Classification of ligninolytic bacteria

Classes of Bacteria	**Ligninolytic Bacteria**	**References**
Actinobacteria	*Streptomyces viridosporus* T7A	Ramachandra *et al.* (1988)
	Rhodococcus sp.	Zimmermann (1990)
	Nocardia autotrophica	Zimmermann (1990)
	Streptomyces coelicolor	Ahmad *et al.* (2010)
	Rhodococcus jostii RHA 1	Ahmad *et al.* (2010)
	Rhodococcus erythropolis	Ahmad *et al*. (2010)
	Arthrobacter globiformis	Ahmad *et al.* (2010)
	Micrococcus luteus	Taylor *et al*. (2012)
	Microbacterium phyllosphaerae	Taylor *et al.* (2012)
	Miicrobacterium oxydans	Taylor *et al.* (2012)
	Microbacterium marinilacus	Taylor *et al.* (2012)
	Nonomuraea gerenzanensis	Casciello *et al.* (2017)
α-Proteobacteria	*Sphingobium* sp. SYK-6	Masai *et al.* (2007)
	Ochrobactrum rhizosphaerae	Taylor *et al.* (2012)
	Ochrobactrum pseudogrignonense	Taylor *et al.* (2012)
	Mesorhizobium sp. PT04.	Tian *et al.* (2016)
	Ensifer adhaerens NWODO-2	Falade *et al.* (2017b)
γ-Proteobacteria	*Pseudomonas putida* mt-2	Ahmad *et al.* (2010)

	Acinetobacter sp.	Ahmad *et al.* (2010)
	Raoultella ornithinolytica S12	Bao *et al.* (2015)
	Serratia liquefacien PT01	Tian *et al.* (2016)
	Pseudomonas chlororaphis PT02	Tian *et al.* (2016)
	Stenotrophomonas maltophilia PT03	Tian *et al.* (2016)
	Raoultella ornithinolytica OKOH-1	Falade *et al.* (2017b)
Bacilli	*Bacillus* sp. LD003	Bandounas *et al.* (2011)
	Bacillus sp. CS-1	Chang *et al.* (2014)
	Bacillus sp. CS-2	Chang *et al.* (2014)
	Bacillus ligniniphilus L1	Zhu *et al.* (2017)

2.3. Ligninolytic enzymes

Ligninolytic activities of microbes have been partly attributed to their ability to produce potent extracellular oxidative enzymes (Tien and Kirk, 1983; Glenn *et al.,* 1983) known as lignin modifying extracellular enzymes (LMEs). Ligninolytic enzymes have been broadly classified into phenol oxidases and heme peroxidases. Enzymes in the phenol oxidases include laccases (EC 1.10.3.2) while the heme-peroxidases include lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and dyP-type peroxidases (EC 1.11.1.19). Also implicated in the degradation of lignin are some accessory enzymes such as aryl-alcohol oxidase (EC 1.1.3.7), glyoxal oxidase (EC 1.2.3.5) and glucose 1-oxidase (EC 1.1.3.4) which generate the hydrogen peroxide (H_2O_2) required by the peroxidases (Kersten and Kirk, 1987; Guillen *et al.,* 1992; Ander and Marzullo, 1997).

LMEs have also shown a capability towards the degradation of various xenobiotics including dyes, chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorous compounds and phenols (Wesenberg *et al.,* 2003). The high redox potentials of ligninases and their ability to oxidize materials recalcitrant to degradation motivates for their prospects in biopulping and biobleaching (Call and Call, 2005), bioremediation through textile dye transformation (Husain, 2010; Mehta, 2012), decolourization of distillery effluent and other waste effluent treatment (Rajasundari and Murugesan, 2011) and as well, the degradation of herbicides (Pizzul *et al.,* 2009). Consequently, the interest in the application of ligninases for biotechnological purposes continues and the imperativeness of the industrial potential is an indication of the value of these enzymes.

2.4. Peroxidases

Peroxidases catalyse the oxidation of various organic and inorganic substrates in the presence of hydrogen peroxide as electron acceptor; a typical reaction is as shown below.

$$2S + H_2O_2 + 2e^- \longrightarrow 2S_{ox} + 2H_2O,$$

S ≈ substrate (electron donor), S_{ox} ≈ oxidized substrate.

Peroxidases are distributed widely in nature with vast presence in plants, animals and microbes having been documented (Battistuzzi *et al.,* 2010). They are grouped as heme and non-heme peroxidases. The heme peroxidases contain a protoporphyrin IX (heme) as prosthetic group while the non-heme peroxidases lack such prosthetic group. The report on peroxidase sequences which is available in PeroxiBase indicates that the majority of the sequences (over 70%) encode heme-peroxidases (Passardi *et al.,* 2007; Zamocky and Obinger, 2010). This observation suggests heme-containing peroxidases as the most abundant as well as most prominent in nature.

A recent classification phylogenetically divides heme peroxidases into two superfamilies, (peroxidase-cyclooxygenase superfamily and peroxidase-catalase superfamily) and three families composed of di-heme peroxidases, dyP-type peroxidases (DyPPrx) and haloperoxidases (HalPrx) respectively (Zamocky and Obinger, 2010).

The peroxidase-cyclooxygenase superfamily is made up of members from all domains of life (Zamocky *et al.,* 2015) as against the old nomenclature “animal heme-dependent peroxidases” which formerly restricted classification to only peroxidases of animal origin. This superfamily seems to dominantly catalyse halide oxidation (Zamocky *et al.,* 2015). Several representatives of peroxidase-cyclooxygenase superfamily are involved in the innate immune system (Söderhall, 1999). This function is not restricted to mammalian peroxidases alone as several peroxidases of bacterial origin (Dick *et al.,* 2008) are suspected to be involved in unspecific defence mechanisms (Zamocky and Obinger, 2010). The involvement of the peroxidase-cyclooxygenase superfamily in immunology would be of clinical significance.

The peroxidase-catalase superfamily may be further subdivided into three classes (Welinder, 1992). Class I involves intracellular peroxidases such as yeast cytochrome c peroxidase (CcP) which protects against toxic peroxide in the electron transport chain (Dunford, 1999), recent

evidences also suggest that this peroxidase functions as a mitochondrial peroxide sensing and signaling protein in *Saccharomyces cerevisiae* (Martins *et al.,* 2013). Ascorbate peroxidase (APx) comes next, and it is associated with the removal of hydrogen peroxide in the chloroplast and cytosol of higher plants (Dunford, 1999; Battistuzzi *et al.,* 2010) and lastly, the bacterial catalase-peroxidase (KatG) which is known to exhibit hybrid catalytic activities of both peroxidase and catalase and is thought to have cell protective fender under oxidative stress (Smulevich *et al.,* 2006; Battistuzzi *et al.,* 2010; Welinder, 1992). Class II, of the peroxidase-catalase superfamily, are extracellular fungal peroxidases including lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) which are involved in lignin degradation while class III includes peroxidases secreted by plants such as horseradish peroxidase (HRP) which have been implicated in cell wall biosynthesis, Indole-3-acetic acid catabolism and oxidation of poisonous compounds (Veitch and Smith, 2001; Battistuzzi *et al.,* 2010).

2.5. Ligninolytic peroxidases

Class II heme-peroxidases including MnP, LiP and VP are the major peroxidases implicated in ligninolysis and are reported as fungal or bacterial in nature. They are extracellular enzymes associated with lignin degradation and, perhaps, portend vital roles in the valorization of lignocellulosic biomass to commercializable products. Class II heme-peroxidases play a central role in delignification (Ruiz-Dueñas and Martinez, 2009). These peroxidases; MnP, LiP and VP, oxidize specific components of the lignin structure and may act in synergy if they are produced by the same organism. While MnP oxidizes the phenolic structures of lignin and LiP targets the non-phenolic components, VP has the capability of oxidizing both phenolic and non-phenolic structures. Moreover, lignin-degradation potential of DyP-type peroxidases and catalase-peroxidase have recently been reported (de Gonzalo *et al.,* 2016).

2.5.1. Manganese peroxidase (EC.1.11.1.13)

MnP was discovered by Kuwahara *et al.* in 1984 and has been described as the most common lignin-modifying peroxidase secreted by most white-rot fungi and litter decomposers (Hofrichter, 2002). Its involvement in lignin degradation has been reported and well-studied in fungi (Hofrichter, 2002), however; paucity of information exists on MnP-producing bacteria. The mechanism of action of MnP includes the catalytic oxidation of Mn^{2+} to Mn^{3+}, which is highly

reactive and in turn oxidizes a wide range of phenolic substrates including lignin phenolic structures (Tuor *et al.,* 1992). The Mn^{3+}, formed from the oxidation of Mn^{2+} present in lignocellulosic materials is stabilized by reacting with a carboxylic acid such as tartrate which serves as ion chelator. The resultant complex will in turn oxidize the phenolic component of lignin structure which leads to generation of unstable radicals that may breakdown naturally (Hofrichter, 2002). Nonetheless, MnP also possesses the capability to oxidize or cleave non-phenolic structures with the contributions of mediators including thiyl or lipid radicals (Reddy *et al.,* 2003a; Abdel-Hamid *et al.,* 2013). Moreso, the ability of MnP to oxidize and depolymerize natural and synthetic lignin and as well, recalcitrant compounds has been reported (Dehorter and Blondeau, 1993; Bogan *et al.,* 1996, Hofrichter *et al.,* 2001; Hofrichter, 2002; Hofrichter *et al.,* 2010). MnPs possess two or three residues corresponding to Glu^{35}, Glu^{39} and Asp^{175} of *Phanerochaete chrysosporium* MnP 1 that binds Mn (Ruiz-Dueñas *et al.,* 2009; Floudas *et al.,* 2012).

Generally, MnP has a molecular weight range of 38 to 62.5 kDa, with most purified MnPs having molecular weights of about 45 kDa (Hatakka, 1994). About 11 various isoforms of MnP have been identified in *Ceriporiopsis subvermispora* (Lobos *et al.,* 1994) with variations in the isoelectric point of the different isoforms.

2.5.2. Lignin peroxidase (EC 1.11.1.14)

Lignin peroxidase (LiP) is also referred to as diaryl propane oxygenase and is a heme-containing enzyme that catalyzes hydrogen peroxide-dependent oxidative degradation of lignin (Fig. 2.1). Ligninase I, similarly serves the same function as diaryl propane peroxidase. These enzymes are inclusive of the peroxidase-catalase superfamily (Zamocky and Obinger, 2010). Structurally, LiP is a monomeric hemoprotein. The non-planarity of the heme cofactor of LiP and those in the other class-II peroxidases has been well documented (Piontek *et al.*, 1993), and observable in the structures of the different ligninolytic peroxidases deposited in the Protein Data Bank (PDB).

After the discovery of LiP in extracellular medium of white-rot fungus; *P. chrysosporium* (Tien and Kirk, 1983; Glenn *et al.,* 1983), various isozymes have been identified in the following organisms: *P. chrysosporium* (Farrell *et al.,* 1989), *Tramates versicolor* (Johansson *et al.,* 1993), *Phlebia radiata* (Moilanen *et al.,* 1996) and *Phanerochaete sordida* (Sugiura *et al.,* 2009). Farrell *et al.* (1989) demonstrated the existence of six (6) isozymes of LiP designated H1, H2, H6, H7, H8 and H10 in the extracellular fluid of cultures of *P. chrysosporium* BKM-F-1767. Another

isozyme of lignin peroxidase, Ha was later identified by Dass and Reddy (1990). In the same vein, Glumoff *et al.* (1990) also characterized five (5) isozymes of LiP from *P. chrysosporium*. The study reported that the purified isozymes had different isoelectric points, sugar content, substrate specificity and stability. The N-terminal sequences of their amino acids were also reported to be different which suggested that they were encoded by different genes. Gene sequencing of a lignocellulose degrading fungus; *P. chrysosporium* strain RP78, revealed about ten *lip* genes thus confirming the existence of isozymes of LiP (Martinez *et al.* 2004). Furthermore, Morgenstern *et al*. (2008), in consistence with previous studies, reported *P. chrysosporium* genome to harbour ten *lip* genes designated *lip* A-J and which respectively encode different isoforms of lignin peroxidase. The US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov) receives consistent lignocellulolytic fungi genome sequences updates with new genes coding for ligninolytic peroxidases, including different LiP isoforms, having been identified as recent as 2015 (Ruiz-Dueñas *et al.*, 2013; Hori *et al.*, 2014; Couturier *et al.*, 2015).

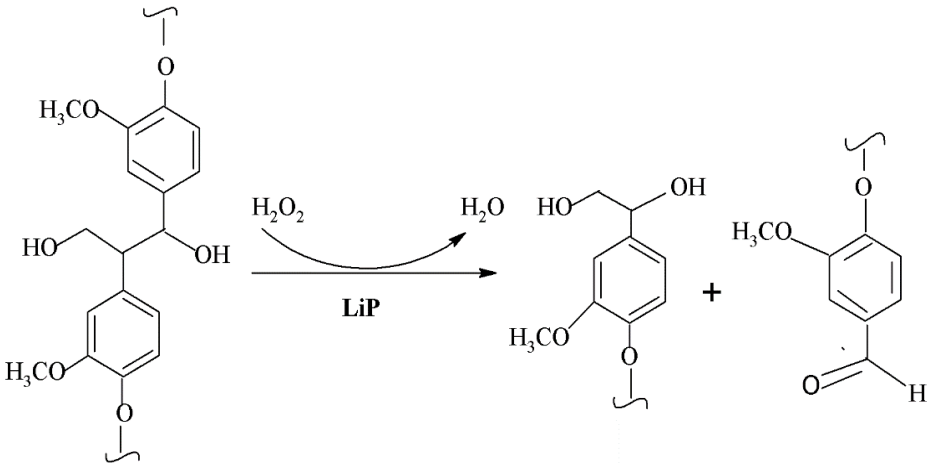

Fig. 2.1. Oxidative cleavage of β- 1 linkage in lignin structure by lignin peroxidase (LiP) (Falade *et al.*, 2017a).

Structurally, LiP folds to form a globular shape with a size of about 50 x 40 x 40 Å (Piontek *et al.,* 1993). It is segregated into proximal and distal domains by the heme which is completely fixed in the protein but made accessible through two small channels. The LiP folding motif contains eight major α-helices, eight minor helices and three short antiparallel β sheets (Choinowski *et al.,* 1999). In the overall, the catalytic cycle of LiP is comparable to that of typical heme-peroxidases. However, some structural variations between lignin peroxidase and other heme-peroxidases exist. Similarly, the molecular weight range of lignin peroxidase has been documented as 38 kDa to 43 kDa with an isoelectric point range of 3.3 to 4.7 (Kirk *et al.,* 1986a; Glumoff *et al.,* 1990) and a very low optimum pH of around pH 3.0 with veratryl alcohol as the substrate (Tien and Kirk, 1988; Furukawa *et al.,* 2014). The low optimum pH of LiP distinguishes it from other peroxidases.

Crystallographic studies of cytochrome c peroxidase (CcP) and LiP revealed some structural differences; LiP possesses four di-sulphide bonds while CcP has none. LiP is larger in size and contains about 343 amino acid residues while CcP is made up of 294 residues (Edwards *et al.* 1993). However, CcP is thought to be abundantly endowed with oxidizable amino acids (7 tryptophans, 14 tyrosine residues, 5 methionines and 1 cysteine) and in contrast, LiP has 3 tryptophans and 8 methionines. Tyrosine is absent in LiP and, it also does not have free cysteine. Nonetheless, a very notable difference between LiP and CcP includes the presence of phenylalanines at the contact point between the distal and proximal heme surfaces in LiP and the replacement of phenylalanines with tryptophans in the case of CcP. Similarly, Asp^{183} is hydrogen bonded to heme propionate in LiP while, Asn^{184} plays this role in CcP. This has been suggested to partly account for the low pH optimum of lignin peroxidase as the disruption of the aspartic acid-propionate hydrogen bond would be expected to result in the destabilization of the heme pocket. The works of Choinowski *et al.* (1999) similarly revealed that the bond between the heme iron and the $N^{\varepsilon 2}$ atom of the proximal histidine residue in LiP is longer than that in CcP. The weaker iron-nitrogen bond in LiP makes the heme more electron deficient thereby destabilizing the high oxidation states which has been suggested as a reasonable explanation for the higher redox potential of lignin peroxidase when compared to cytochrome c peroxidase.

2.5.2.1. Lignin peroxidase catalytic reactions

LiP oxidizes different non-phenolic lignin model compounds including β-O-4 linkage-type arylglycerol-aryl ethers. LiP oxidative properties involve the formation of radical cation through one electron oxidation and, this action leads to side chain cleavage, demethylation, intramolecular addition and rearrangements (Kirk *et al.,* 1986b; Miki *et al.,* 1986; Wong, 2009). Hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to their corresponding aldehydes or ketones and phenol oxidation are other mechanistic oxidative processes associated with LiP (Paliwal *et al.,* 2012; Furukawa *et al.,* 2014).

LiP possesses high redox potential for the oxidation of non-phenolic structures which constitute up to 90% of lignin (Martinez *et al.,* 2005). It is also characterized with the ability to oxidize a wide range of aromatic compounds hence, its role in the enzymatic degradation of lignin. Besides the characteristic oxidation of non-phenolic substrates, LiP has also shown the capability to oxidize a variety of phenolic compounds such as ring- and *N*-substituted anilines (Baciocchi *et al.*, 2001). Guaiacol, acetosyringone, catechol, vanillyl alcohol and syringic acid are other phenolics susceptible to the oxidative potentials of LiP (Harvey and Palmer, 1990; Wong, 2009). At this juncture, it would suffice to state that veratryl alcohol, a non-phenolic metabolite and high redox potential substrate has been suggested as a redox mediator (Christian *et al.,* 2005) as it has been reported to enhance lignin peroxidase activity in lignin degradation (Lundell *et al.,* 1993; Schoemaker *et al.,* 1994). The ability of LiP to oxidize lignin and other high redox potential compounds has been attributed to its exposed tryptophan residue (Trp^{171}) which forms a tryptophanyl radical on the surface of the enzyme through long-range electron transfer (LRET) to the heme. Also, variation in the tryptophan environment has been identified as a factor capable of modulating the enzyme activity, stability and substrate specificity (Ivancich *et al.,* 2001). This, perhaps, accounts for the variation in the catalysis of VP and LiP as LiP is able to oxidize veratryl alcohol more effectively than VP, an ability which can be attributed to the acidic environment of Trp^{171} in *P. chrysosporium* LiP as it facilitates the stabilization of veratryl alcohol cation radical (Khindaria *et al.,* 1996).

The catalytic cycle of lignin peroxidase involves three steps (Fig. 2.2). The first reaction step is the oxidation of the resting ferric enzyme [Fe (III)] by hydrogen peroxide (H_2O_2) as an electron acceptor resulting in the formation of compound I oxo-ferryl intermediate. In the second step, the

oxo-ferryl intermediate (deficient of $2e^-$) is reduced by a molecule of substrate such as non-phenolic aromatic substrate (S) which donates one electron ($1e^-$) to compound I to form the second intermediate, compound II (deficient of $1e^-$) while the last step involves the subsequent donation of a second electron to compound II by the reduced substrate thereby returning LiP to the resting ferric oxidation state which indicates the completion of the oxidation cycle (Abdel-Hamid *et al.*, 2013).

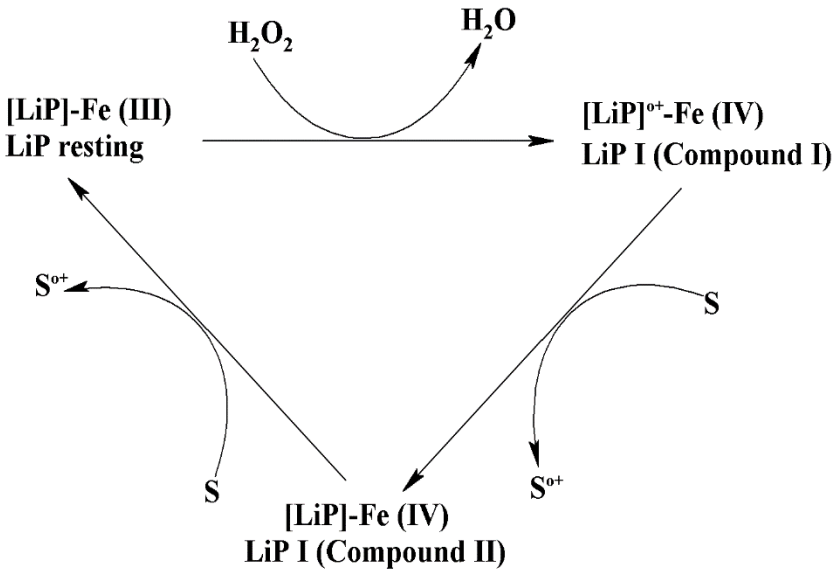

Fig. 2.2. Catalytic reaction of lignin peroxidase. Adapted from Abdel- Hamid *et al.* (2013).

2.5.3. Versatile peroxidase (EC.1.11.1.6)

Versatile peroxidase (VP) is a heme-containing microbial enzyme belonging to class II of peroxidase-catalase superfamily. It is also referred to as "hybrid peroxidase" or "lignin-manganese peroxidase" and is largely produced by ligninolytic fungi belonging to certain genera *Bjerkandera* (Heinfling *et al.,* 1998), *Pleurotus* (Ruiz-Duenas *et al.,* 1999; Palma *et al.,* 2016) and *Lepista* (Zorn *et al.,* 2003). Its production by *Phanerochaete chrysosporium* has also been reported (Coconi-Linares *et al.,* 2014). However, there is dearth of information on its production by bacteria. Thus,

exploitation of bacteria and other ligninolytic fungi strains for versatile peroxidase production is imperative.

VP is a unique ligninolytic enzyme with the ability to combine the substrate specificity properties of two ligninolytic peroxidases (MnP and LiP) and one other fungal peroxidase family, *Coprinopsis cinerea* peroxidase (CIP) (Perez-Boada *et al.,* 2005). Consequently, it is capable of oxidizing a range of both high and low redox potential substrates such as Mn^{2+}, phenolic and non-phenolic lignin model dimers, α-keto-γ-thiomethylbutyric acid (KTBA), veratryl alcohol (VA), dimethoxybenzenes, synthetic dyes, substituted phenols and hydroquinones (Caramelo *et al.,* 1999; Perez-Boada *et al.,* 2005). VP employs the manganese peroxidase (MnP) pathway by oxidizing Mn^{2+} to Mn^{3+} with hydrogen peroxide as electron acceptor (Fig. 2.3); however, Mn^{3+} is highly reactive but has a very short half-life. Consequently, when VP is utilizing the MnP pathway, a dicarboxylic organic acid such as oxalate, tartrate or malonate is required to form a stable complex with Mn^{3+} (Mn^{3+}-oxalate, Mn^{3+}-tartrate or Mn^{3+}-malonate). Thus, with the utilization of this mechanism, versatile peroxidase is capable of oxidizing pollutants situated far away from it by the action of the metallic ion-complex (Toboada-Puig *et al.,* 2015). The utilization of MnP pathway by VP commits it to oxidation of phenolic substrates as it is also able to oxidize non-phenolic compounds and other typical substrates of LiP using the normal LiP catalytic reaction mechanism. However, LiP is able to oxidize veratryl alcohol, a typical LiP substrate more effectively than VP. The variation in the catalysis of LiP and VP has been attributed to the variation in the tryptophan environment of the enzymes (Khindaria *et al.,* 1996).

VP also employs the long-range electron transfer (LRET) mechanism in the oxidation of high redox-potential aromatic compounds (Ruiz-Dueñas *et al.,* 2009). Specifically, three possible LRET pathways for the oxidation of high redox potential aromatic compounds have been revealed in two VP isozymes (VPL and VPS 1) of *Pleurotus eryngii* (Caramelo *et al.,* 1999; Ruiz-Dueñas *et al.,* 1999; Perez-Boada *et al.,* 2005). These pathways start at either Trp^{164} or His^{232} of VPL and at His^{82} or Trp^{170} of VPS 1 which is homologous to Trp^{164} in VPL (Perez-Boada *et al.,* 2005; Ruiz-Dueñas *et al.,* 2009). Furthermore, the involvement of Trp^{164} in the oxidation of veratryl alcohol and reactive black 5 has been reported. However, the other two pathways (His^{232} and His^{82}) were not involved in LRET (Perez-Boada *et al.,* 2005). Therefore, the ability of VP to oxidize high redox potential compounds could perhaps be linked to an exposed catalytic tryptophan: Trp^{164}

which forms a radical on the surface of the enzyme through a LRET to the heme (Ruiz-Dueñas *et al.*, 2009; Saez-Jimenez *et al.*, 2015). Hence, LRET could suffice as a novel mechanism for EDC removal by VP.

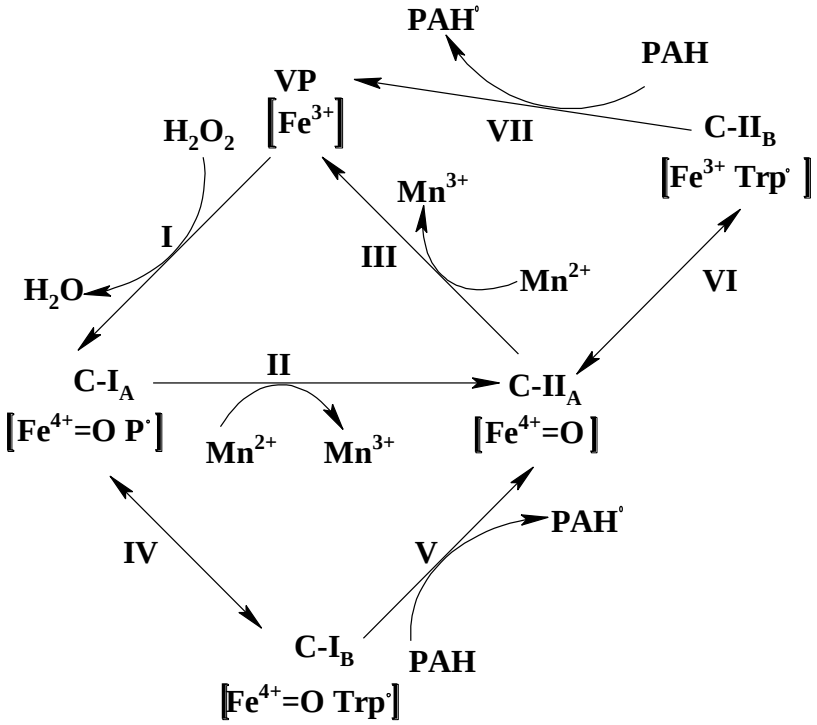

Fig. 2.3. Exploitation of the VP catalytic cycle for EDC removal. The VP catalytic cycle is adapted from Pérez-Boada *et al.* (2005) with permission from Elsevier, licence number: 4016930743990 (Appendix A). C-I_A (Compound I_A, containing Fe^{4+}-oxo and porphyrin cation radical), C-II_A (Compound II_A, containing Fe^{4+}-oxo after reduction of porphyrin), C-I_B (Compound I_B, containing Fe^{4+}-oxo and tryptophanyl (Trp^{164}) radical), C-II_B (Compound II_B, containing Fe^{3+} and tryptophanyl (Trp^{164}) radical), PAH (Polycyclic Aromatic Hydrocarbons). **I-III:** Reactions involved in Mn^{2+} oxidation mechanism proposed for phenolic EDC removal. Mn^{3+} generated in step III forms a complex with a dicarboxylic acid such as oxalate/malonate/tartrate which is responsible for subsequent degradation of phenolic EDC. **I, IV-VII:** Reactions involved in long range electron transfer mechanism proposed for EDC removal. The tryptophanyl radical generated on the surface of C-II_B could be exploited for degradation of non-phenolic EDC such as PAH in step VII.

2.5.4. DyP-type peroxidases (EC.1.11.1.19)

DyP-type peroxidases (dye decolourizing peroxidases) is another family of heme-peroxidases with ligninolytic potential (Sugano, 2009; Colpa *et al.*, 2014; Singh and Eltis, 2015; Yoshida and Sugano, 2015). However, its ligninolytic activity is still under discussion as leading scientists in the field of lignin degradation who are also involved in the study of DyP-type peroxidases (DyPs) consider that a significant contribution of these peroxidases to degradation of lignin is improbable perhaps, due to their "low activity on high redox-potential substrates" (Linde *et al.,* 2015). Nonetheless, several DyP-type peroxidases have been implicated in lignin degradation as well as degradation of lignin model compounds (de Gonzalo *et al.,* 2016).

The first DyP was purified and characterized from a culture of *Bjerkandera adusta* (Kim and Shoda, 1999) misidentified as *Geotrichum candidum* (Ruiz-Dueñas *et al.,* 2011). The name "dye decolourizing peroxidase" was derived from the activity of the enzyme on anthraquinone and azo dyes (Sugano *et al.,* 2007).

DyPs are produced by both fungi and bacteria. Unlike other ligninolytic heme-peroxidases: MnP, VP and LiP, whose production seem to be restricted to fungi, DyPs are found in relatively large amount in bacteria (Van Bloois *et al.,* 2010). More so, a vast number of bacterial DyPs have been reported (Lambertz *et al.,* 2016). This is consistent with the assumption that putative genes encoding DyPs are amply present in the bacterial genome (Van Bloois *et al.,* 2010). DyPs are different from classical peroxidases in primary sequence and structure. On the basis of their sequence characteristics, they are classified into four (A, B, C and D) in the peroxidase database: PeroxiBase (Fawal *et al.,* 2013). Classes A, B and C comprise bacterial DyPs while extracellular fungal DyPs belong to Class D (Yoshida and Sugano, 2015). Class A DyPs are usually extracellular as they possess a Tat-signal sequence which is absent in Classes B and C DyPs, hence they are intracellular (de Gonzalo *et al.,* 2016).

It is worthy of note that DyPs from bacteria seem to possess a lower oxidizing ability than the fungal DyPs as they appear to oxidize only less recalcitrant phenolic lignin model compounds and monophenolic substrates (de Gonzalo *et al.,* 2016). Nevetheless, bacterial DyPs have exhibited significant activity for oxidation of the non-phenolic veratryl alcohol (Santos *et al.,* 2014; Yu *et al.,* 2014; Min *et al.,* 2015). Other substrates of DyPs include Mn^{2+} and β-carotenes but their

physiological substrate is yet unknown (de Gonzalo *et al.*, 2016). In other words, DyPs are characterized by very wide substrate specificity.

Liers *et al.* (2014) compared the oxidation of selected high-redox potential phenolic substrates by DyPs and other types of peroxidases including soybean peroxidase, *Coprinopsis cinerea* peroxidase and lignin peroxidase. The study observed that DyPs possess a high affinity for recalcitrant phenolic compounds such as *P*-nitrophenol. More so, a redox potential ranging between 1.10 ± 0.02 V and 1.20 ± 0.1 V was estimated for the DyPs studied. Interestingly, the estimated redox potential for DyPs was comparable to that of lignin peroxidase (1.26 ± 0.17V), which is regarded as a high redox potential enzyme. It is worthy of note that oxidation of phenolic compounds by DyPs do not necessitate the use of a redox-mediator as required by lignin peroxidase and manganese peroxidase (Liers *et al.*, 2014). Therefore, their catalytic efficiency in this regard, resembles that of versatile peroxidase (Martinez *et al.*, 1996). As in LiP and VP, DyPs employ the LRET pathway for its oxidation process as recent studies have identified Tyr^{337} (Strittmatter *et al.*, 2013), Tyr^{391}, Tyr^{403} and Tyr^{388} (Liers *et al.*, 2014) as amino acid residues that play the role of Trp^{171} and Trp^{164} in LRET pathways of *P. chrysosporium* LiP and *P. eryngii* VP respectively.

DyPs are majorly active in an acidic environment (acidic pH) and possess a molecular weight of about 40-60 kDa (de Gonzalo *et al.*, 2016). Structurally, they are not similar to the common peroxidases of fungal origin, nevertheless, they are related in terms of their catalytic reactions. In spite of the structural variation, the physical and chemical properties of DyPs are comparable to those of typical heme peroxidases (Liers *et al.*, 2010).

2.5.5. Catalase-peroxidase (EC.1.11.1.21)

Catalase-peroxidases (KatGs) belong to Class I peroxidases of the peroxidase-catalase superfamily of heme-peroxidases (Zamocky and Obinger, 2010), also known as the superfamily of plant, bacterial and fungal heme-peroxidases (EC 1.11.1.7). The first catalase-peroxidase was reported as hydroperoxidase I (HPI) from *Escherichia coli* and was originally categorized as a catalase with a wide range of peroxidase activity (Claiborne and Fridovich, 1979; Singh *et al.*, 2008). Further characterization of the gene encoding HPI (*katG*) showed similarity to plant peroxidases (Triggs-Raine *et al.*, 1988). Hence, its categorization in Class I peroxidases. Nevertheless, KatGs are either

homodimers or homotetramers of approximately 80 kDa subunits which is different from typical peroxidases which exist as monomers (Singh *et al.*, 2008). They are exceptional bifunctional enzymes found in both prokaryotes and eukaryotes.

The phylogenetic analysis of *katG* gene sequences in PeroxiBase by Passardi *et al.* (2007) showed that the genes were found in approximately 40% of bacterial genomes. The study also observed that, occasionally, species that are closely related differ as they possess *katG* genes of diverse sources or lack any catalase peroxidase gene. In eukaryotes, *katG* genes exist in different evolutionary lines: algae and fungi lines. However, both evolutionary lines are likely to have originated from horizontal gene transfer from bacteria (Passardi *et al.,* 2007; Zamocky *et al.,* 2007).

KatGs are unique bifunctional enzyme characterized by predominant catalase activity and significant peroxidase activity (Zamocky *et al.,* 2008). Although KatGs are like other members of class I (cytochrome c peroxidases and ascorbate peroxidases) in their sequences and structures, they are the only heme-peroxidase with the ability to reduce and efficiently oxidize hydrogen peroxide at the same rate as a "typical" (monofunctional) catalases (Vlasits, 2009). They oxidize typical peroxidase substrates including pyrogallol, guaiacol, *o*-dianisidine and ABTS [2-2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)]. However, the naturally occurring peroxidase substrate is not known. Biochemical characterization of KatG showed that its peroxidase activity was optimum at around pH 5.5 (Vlasits, 2009). Moreover, KatGs have also been reported to have "halogenation" (Jakopitsch *et al.,* 2001) and "NADH oxidase" (Singh *et al.,* 2004) activity. In spite of the number of reactions catalyzed by this enzyme, its actual physiological function, besides removal of hydrogen peroxide, is not clear and has remained unknown (Singh *et al.,* 2008). Nonetheless, catalase-peroxidase from *Mycobacterium tuberculosis* has been reported to activate isoniazid, an anti-tuberculosis drug (Zhao *et al.,* 2006) in physiological conditions.

Interestingly, a recent review has implicated bacterial catalase-peroxidases in lignocellulose degradation (de Gonzalo *et al.,* 2016). Brown *et al.* (2011) used a proteomic approach to observe the secretion of catalase-peroxidase (Amyco 1) by *Amycolatopsis* sp. 75iv2 when incubated with lignocellulosic material. To confirm the involvement of Amyco 1 in lignin degradation, the secreted enzyme was produced using recombinant technology and purified. The study revealed that Amyco 1 was able to convert a phenolic lignin-model compound. The ability of catalase-

peroxidase from *Amycolatopsis* sp. 75iv2 to act on lignin-like compound indicates its potential in lignin modification. However, further research is required to ascertain the involvement of bacterial catalase-peroxidases in lignin degradation.

2.6. Peroxidase functionalities and prospective applications

2.6.1. Contemporary and prospective functionalities of peroxidases

Generally, peroxidases have been applied in soil detoxification (Mougin *et al.,* 1994), treatment of phenols and chlorophenols polluted wastewaters (Duarte-Vazquez *et al.,* 2003; Cheng *et al.,* 2006), biopulping and biobleaching (Hatakka *et al.,* 2003), development of biosensors to determine the presence of hydrogen peroxide and other related compounds (Jia *et al.,* 2002; Hamid and Khalil, 2009) and in the development of skin-lightening cream (Draelos, 2015). Most of these applications are yet to be commercialized. Worthy of note is the fact that biopulping, which is regarded as an effective alternative to chemical and mechanical pulping, is one of the oldest applications of peroxidases (Koshy and Nambisan, 2011). Catabolizing lignin in processed wood for paper production is the major role of LiP and other lignin modifying enzymes in the pulp and paper industry (Michael *et al.,* 1991; Jaspers *et al.,* 1994).

Recently, the applications of peroxidases have extended to development of cosmeceutical and dermatological products. Most notable of these products are Melanozyme^{TM} (lignin peroxidase based product) which is marketed as "*elure^{TM} skin brightening cream*" (www.elureskin.com) and Luminase, which serves as a catalytic skin tone illuminator, both manufactured by Syneron Medical Ltd, Irvine, California, USA for the treatment of hyperpigmentation (sun spots or age spots) and skin lightening. The LiP used in the development of these skin lightening products has solely been derived from *P. chrysosporium*.

The potential applications of peroxidases in various other sectors have been envisaged (Hamid and Khalil, 2009), and interests in further exploit of these enzymes for industrial applications are on the increase. The, supposedly, high redox potential which bestows the desired functionality has been the reason for the endeared interest (Maciel *et al.,* 2010). In the light of this knowledge, it becomes obvious that the prospective applications of peroxidases span through vast sectors of human endeavour including the bio-refinery, textile, bioremediation, cosmetology and dermatology. An overview of some functionalities is presented in succeeding sections.

2.6.2. Delignification of feedstock for ethanol production

Ethanol is a good alternative to fossil fuel and as such, the use of lignocellulosic biomass as cheap source of feedstock for production of ethanol has continued to receive attention globally due to, in part, their renewable and eco-friendly nature. Delignification of lignocellulose is an imperative step in the bioconversion of lignocellulose to ethanol and this process remains a challenge in lignocellulose biomass valorization. A biological method of delignification has been suggested as promising due to its mild reaction conditions, higher product yield and low energy demand (Sánchez *et al.,* 2011). But on the downside, it is saddled with long incubation period (in the order of several weeks to months) before reaching the same product (cellulose) recovery as it's obtained with the physical and chemical pre-treatment methods (Khuong *et al.,* 2014). Additionally, the utilization of carbohydrate as carbon source by the de-lignifying microbes has been shown to impact adversely on the quantity of recovered products (Sun *et al.,* 2011).

In a bid to overcome some of the challenges associated with microbial mediated bio-conversion of lignocellulose, novel "lignocellulolytic enzyme system" has been suggested as an effective treatment strategy (Mukhopadhyay *et al.,* 2011; Wang *et al.,* 2013). The suggested lignocellulolytic enzyme system includes LiP, MnP and laccase among others and the associated merits of reaction specificity and, high product yield occasioned by the non-utilization of products as source of energy (Wang *et al.,* 2013; Ma and Ruan, 2015). This makes the system a very promising model for industrial application. To buttress this position, Asgher *et al*. (2013) showed that ligninolytic enzymes (LiP, MnP and Laccase) isolated from *P. ostreatus* IBL-02 exhibited appreciable performance in sugarcane bagasse delignification as compared to sodium hydroxide (NaOH). However, the delignification functions attributed to the ligninolytic enzymes system (LiP, MnP and laccases) from *P. ostreatus*, by Asgher *et al.* (2013), may not be associated to LiP as this fungus does not possess *lip* genes in its genome (Ruiz-Dueñas *et al.,* 2011). However, if the position is to hold true, then, *lip* coding genes associated with the ligninolytic enzymes system shown by *P. ostreatus* would have been plasmid born. Otherwise, the delignification effect can only be attributed to laccases, MnP and VP with MnP/LiP hybrid catalytic properties (Fernandez-Fueyo *et al.,* 2014).

2.6.3. Textile effluent treatment and dye decolourization

The textile industry consumes synthetic dyes significantly (Singh *et al.,* 2015), and these dyes are major sources of environmental pollution. Synthetic dyes such as azo, diazo, acidic, basic, reactive, disperse, metal-complex and anthraquinone-based dyes are divers in structural variability (Christian *et al.,* 2005). Understandably so, estimates of about 10-15% of dyes are lost in water during the process of textile dyeing (Asad *et al.,* 2007; Yanto *et al.,* 2014). Subsequent release as effluent into various environments has also been estimated to amount to about 2-20% thereby portending a huge threat to public health (Yanto *et al.,* 2014). To further highlight the danger posed by these textile dyes in the environment; many of these dyes and their degradation products have been declared toxic (Xu *et al.,* 2007; Singh *et al.,* 2015). Hence, their presence in the environment should be a major concern. Therefore, effective and efficient removal strategy in the environment should be imperative. Consequently, various methods for dye decolourization and treatment of textile effluents have been developed. Some of these methods include adsorption, chemical treatment, ion-pair extraction, coagulation and flocculation techniques (Singh *et al.,* 2015). These methods are effective but also costly and, they generate a great amount of sludge which may eventually create secondary pollution problem (Parshetti *et al.,* 2012). On the converse, biological methods for dye treatment and removal including the use of microbes and macro-molecular structures (enzymes) have been effective and are saddled with less limitation. Studies on the applications of fungi and bacteria in dye abasement abound (Singh and Pakshirajan, 2010; Kumar and Sumangala, 2011; Shah *et al.,* 2013; Singh *et al.,* 2014) however, little attention has been given to the oxidative extracellular enzymes as an independent acting entity thus, against this backdrop Ollikka *et al.* (1993) investigated the ability of some lignin peroxidase isozymes, isolated from *P. chrysosporium*, to decolourize azo, triphenyl methane, heterocyclic and polymeric dyes in comparison with crude enzyme. The capability of the isolated isozymes of lignin peroxidase [LiP 4.65 (H2), LiP 4.15 (H7) and LiP 3.85 (H8)] to decolourize the test dyes in the presence of veratryl alcohol as a mediator was comparable to that of the crude enzyme which exhibited over 75% decolourization rate on the dyes. In another study by Abadulla *et al.* (2000), the ability of enzyme preparations from some fungi (*Pleurotus ostreatus*; *Schizophyllum commune*; *Sclerotium rolfsii*; *Neurospora crassa*; *Polyporus* sp; *Trametes villosa*; and *Myceliophtora thermophile*) to decolourize a range of structurally different dyes was evaluated. It was discovered that the enzyme preparations effectively decolourize azo, triarylmethane, anthraquinone and indigo dyes.

Interestingly, the presence of lignin peroxidase increased the rate of decolourization by laccase in the study. Ferreira-Leitao *et al.* (2007) compared the efficiency of fungal lignin peroxidase and plant horseradish peroxidase (HRP) for decolourization of methylene blue and its demethylated derivatives. It was shown that both enzymes were able to oxidize methylene blue and its derivatives. However, lignin peroxidase was reported to be more effective as its oxidation potential was almost double that of HRP. The authors (Ferreira-Leitao *et al.,* 2007) suggested that lignin peroxidase would be more suitable for degradation of phenothyazine dyes and decolourization of wastewater. Also, Shakeri and Shoda (2008) reported the decolourization of an anthraquinone dye, Remazol Brilliant Blue R (RBBR) by a recombinant dye decolourizing peroxidase. Moreover, a lignin peroxidase produced from sewage sludge treatment plant was reported to exhibit potential for textile effluent treatment and dye decolourization (Alam *et al.,* 2009; Singh *et al.,* 2015). This was corroborated by Singh and Pakshirajan (2010) who attributed the high potential of *P. chrysosporium* in decolourization of coloured wastewaters to efficient peroxidase enzyme system. In a study by Parshetti *et al.* (2012), purified peroxidase from *Kocuria rosea* MTCC 1532 decolourized eleven (11) different dyes belonging to various structural groups: azo, triphenyl-methane, heterocyclic, polymeric and metal-complexes. Likewise, Osuji *et al.* (2014) reported the efficient decolourization of Vat Yellow 2, Vat Orange 11 and Vat Black 27 by partially purified peroxidase. Furthermore, detoxification and decolourization of industrial waste by oxidative enzymes from bacteria and fungi have been reported (Rajasundari and Murugesan, 2011). The enzymes oxidize phenolic compounds to aryl-oxy radicals creating insoluble complexes (Abdel-Hamid *et al.,* 2013). Other mechanisms of action of these enzymes include polymerization of contaminants and/or copolymerization with other non-toxic substrates to promote easy removal of the contaminants by other purification methods such as sedimentation, filtration and adsorption (Gainfreda *et al.,* 2006). This further indicates the potential of peroxidases and other oxidative enzymes in textile and other industrial effluent treatment.

2.6.4. Coal depolymerisation and degradation of other xenobiotics

The ligninolytic enzyme system of microbes has been implicated in the degradation of several xenobiotics including chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorus and phenols (Wesenberg *et al.,* 2003; Tisma *et al.,* 2010; Marco-Urrea and Reddy, 2012). These compounds which are released from different anthropogenic sources are

categorised as major environmental pollutants. Some of these compounds are active components of pesticides, disinfectants, herbicides, explosives and dyes among others which are found in daily industrial application. Consequently, accumulation in the soil, ground water and air constantly contaminates the environment and portend nuisance to public health (Wesenberg *et al.,* 2003). Therefore, effective removal of these environmental pollutants is of utmost importance to stakeholders as well as the imperativeness for environmental health. Worthy of note is that extracellular peroxidases, from ligninolytic microbes, have been reported to play a significant role in the degradation of xenobiotic compounds (Duran *et al.,* 2002). LiP, as part of the ligninolytic enzyme system of both fungi and bacteria, has been reported to mineralize different types of recalcitrant aromatic compounds including three- and four-ring polycyclic aromatic hydrocarbons (Gunther *et al.,* 1998; Wesenberg *et al.,* 2003), polychlorinated biphenyl (Krcmar and Ulrich, 1998; Wesenberg *et al.,* 2003), chlorophenols (Antonopoulos *et al.,* 2001) and synthetic dyes (Chivukula *et al.,* 1995; Wesenberg *et al.,* 2003). These articulated data show the suitability of peroxidases in bioremediation.

Furthermore, studies have shown that unburnt coal can have negative effect on water quality and the functioning of the aquatic ecosystem (Ahrens and Morrisey, 2005). Countries in the categorization of large coal producing, consuming and/or exporting are confronted with the challenge of managing the impact of coal in the environment. The presence of coal in water has been suggested as a source of increased salinity, acidity, trace metals, hydrocarbons and chemical oxygen demand (Milani *et al.,* 1999; Ward, 2002; Stephan, 2010). Toxic polycyclic aromatic hydrocarbons (PAHs) from unburnt coal have also been suggested as an important source of contamination in the aquatic environment (Achten and Hofmann, 2009). PAHs from the incomplete combustion of coal have been implicated in the pollution of abandoned coal gasification site (Sutherland *et al.,* 1995) and soil pollution. They are also found at high concentration in the bottom of sediments of water bodies. The presence of coal particles in soils and sediments can result from coal mining and transportation (Johnson and Bustin, 2006; Achten *et al.,* 2011). Given the increased coal mining operations in coal-producing and consuming countries; toxic PAHs as contaminants in water, sediments and soil are continually emerging. Consequently, investigation into the potentials of peroxidases and other ligninolytic enzymes in the degradation of polycyclic aromatic hydrocarbons, known and emerging in the environment

should be explored. Perhaps, of great interest shall be the depolymerisation of PAHs emanating from coal industrial applications and utilizations.

2.6.5. Melanin oxidation - novel cosmetic lightening agents

Melanin, produced by melanocytes in a process termed melanogenesis and stored in melanosomes, is the dark pigment responsible for human skin and hair colouration. The melanosomes are transferred to keratinocytes (epidermal cells) for onward transportation to the upper layer of the epidermis to confer on the skin its typical colour (Mauricio *et al.,* 2011). Its deficiency can lead to various diseases and disorders including albinism which is the absence of melanin pigment in an individual. Pigmentation disorders such as hyperpigmentation, a common dermatologic condition (Kindred *et al.,* 2013) that affects all skin types, have been attributed to the accumulation of melanin in the upper layer of the epidermis (Simon *et al.,* 2009; Mauricio *et al.,* 2011). Basically, melanin is divided into two types: eumelanin (brown and black) and pheomelanin (red or yellow). Eumelanin is reported to be the more ubiquitous melanin type in mammals, as it is found in different parts of the body such as hair, skin, inner ear, eye and brain (Khammuang and Sarnthima, 2013).

The impact and distribution of melanin is not limited to mammals, it is also found in many other life forms including plants and microbes where they serve different functions. Some of the reported biological functions of melanin include protection against environmental stress (Kogej *et al.,* 2007; Liu and Nizet, 2009); increased antibiotic resistance in bacteria (Lin *et al.,* 2005); and involvement in fungal pathogenesis of plants (Butler *et al.,* 2005; Khammuang and Sarnthima, 2013). Melanin is known to be very durable and its durability has been attributable to its complex structure. Its basic structural unit is represented by covalently linked indoles. In addition, melanin is a heterogenous polymer composed majorly of dihydroxyindole units which exist as a mixture of both catechol and quinone (Prota, 1992; Woo *et al.,* 2004). The structural characteristics of melanin are comparable to those of lignin and coal wherein the polymers are made up of indole and phenolic subunits (Woo *et al.,* 2004) hence its resistance to degradation.

Although one of the biological functions of melanin in humans may be to protect the underlying tissues from harmful ultra violet (UV) radiation (Krol and Liebler, 1998), many hyper-pigmented women in Africa and other black nations desire a light face and skin as the Caucasians desire a

spotless skin. To achieve this desire, cosmeceutical and dermatological industries have developed treatments for skin lightening employing the following mechanisms of action: prevention of melanogenesis by inhibiting tyrosinase, an enzyme that catalyzes the rate-limiting step [conversion of tyrosine to dihydroxyphenylalanine (DOPA)] in melanin biosynthesis (Kim and Uyama, 2005) as illustrated in Fig. 2.4, preventing the stimulation of melanocytes by ultraviolet A radiation, activation of cell turn-over (Woo *et al.,* 2004) and blocking the transfer of melanosomes to keratinocytes (Mauricio *et al.,* 2011).

Hydroquinone, described as the gold standard in the treatment of hyperpigmentation (Kindred *et al.,* 2013) has been the most effective skin-lightening agent. However, its safety has generated a lot of controversy and concern. This has motivated research into exploration of alternative agents for the treatment of skin pigmentation disorders including melasma. Currently, some of the available alternative skin-lightening agents include mequinol, topical retinoids, azelaic acid, arbutin and deoxyarbutin, kojic acid, licorice extract, ascorbic acid, soy, aleosin, niacinamide and *N*-acetylglucosamine (Kindred *et al.,* 2013).

Hydroquinone and most of these alternatives operate through tyrosinase inhibition mechanism (Grime, 2009), probably by binding directly to the enzyme or interacting with the copper molecules at its active site (Sheth and Pandya, 2011) thereby reducing the conversion of DOPA to melanin. However, skin-lightening by inhibition of melanin synthesis is slow in achieving the desired results (Woo *et al.,* 2004). Hence, there is the need to explore alternative agents with the potential to directly decolourize melanin pigment through oxidation as a means of skin-lightening potential. Perhaps, the ability of ligninolytic enzymes to oxidize a wide range of structurally different substrates makes them suitable candidates for the oxidation of melanin which is structurally similar to lignin. Thus, ligninolytic enzymes with melanolytic ability have the potential for application in the cosmetics industry.

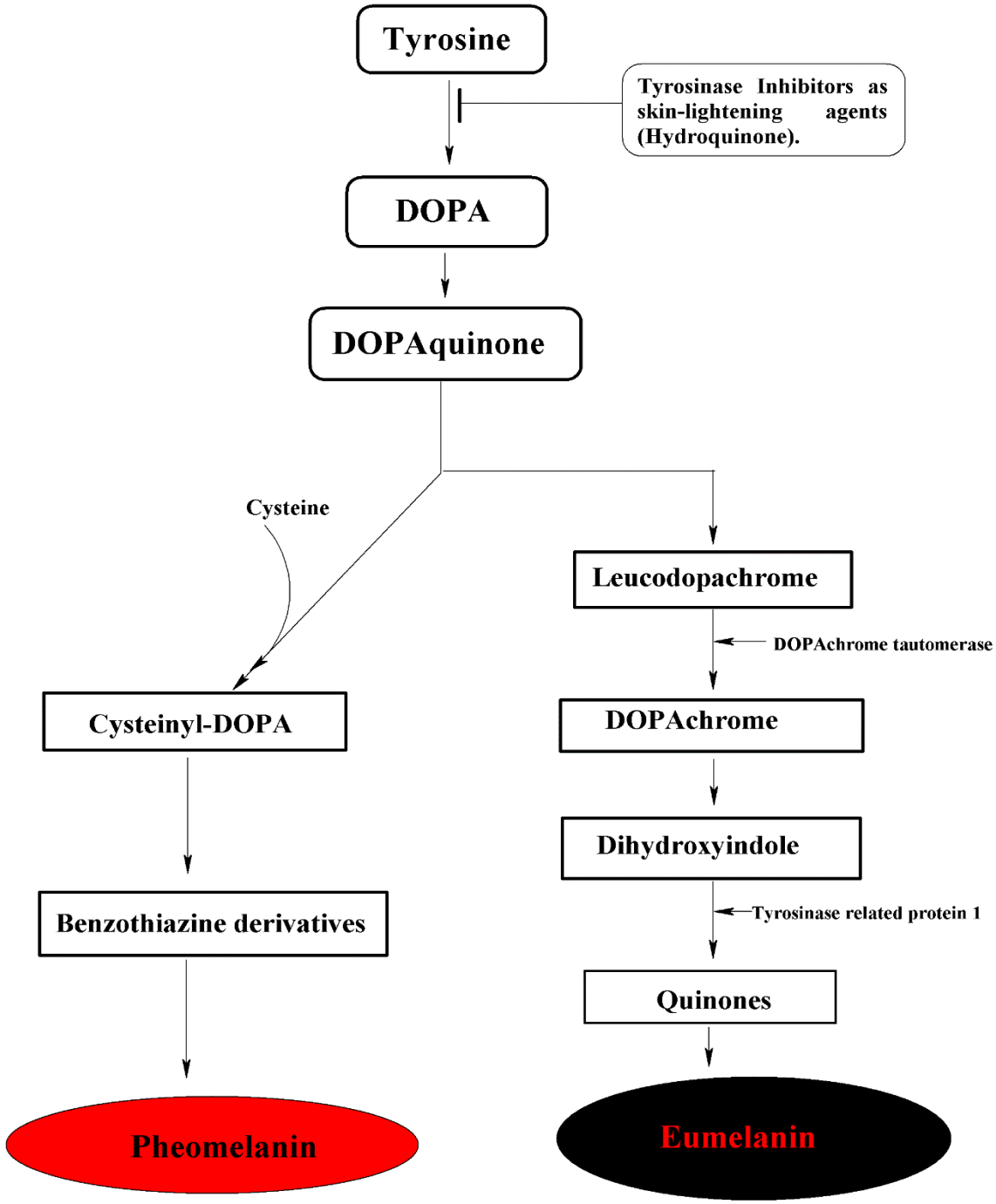

Fig. 2.4. Pathway of melanin biosynthesis (Falade *et al.*, 2017a).

Woo *et al.* (2004) demonstrated that crude peroxidase from *P. chrysosporium* could decolourize synthetic melanin thus suggesting its application in the development of new cosmetic lightening agents. Furthermore, Mohorčič *et al.* (2007) produced melanolytic enzyme capable of degrading human skin melanin from *Sporotrichum pruinosum*. Peroxidases from *Ceriporiopsis* sp. strain MD-1 have also been reported to decolourize synthetic and human hair melanins (Nagasaki *et al.*, 2008). Similarly, the study reported by Khammuang and Sarnthima (2013) showed that crude laccase from *Lentinus polychrous* Lév. was able to decolourize synthetic melanins. The enzyme was reported to be more effective in the presence of ABTS as a mediator. Perhaps, it would be noteworthy to state that all the previously studied melanolytic enzymes are of fungal origin thus, an exploration into bacterial melanolytic enzymes for application in the development of skin care products shall be a novel concept.

As the proposition for the use of lignin peroxidase as an alternative to hydroquinone cream increases, efforts are being made to ascertain efficacy and safety of these compounds both at an acute and chronic phase. Consequently, Mauricio *et al.* (2011) evaluated the skin-lightening efficacy and safety of lignin peroxidase (LiP) constituted cream in comparison with 2% hydroquinone cream in Asian women. It was observed in the study that the application of LiP cream provided a significantly faster and observable skin-lightening effect than 2% hydroquinone cream which led to the overall preference of LiP creams. LiP has demonstrated a skin-lightening effect comparable to that of hydroquinone, with no observable adverse effect, and with superiority in skin texture and roughness (Draelos, 2015). However, more studies are required to compare LiP based cream with higher concentrations of hydroquinone and its efficiency in the treatment of other pigmentary disorders. The mechanism of action of LiP as cosmetic lightening agent involves five steps (Fig. 2.5). The first reaction step is the oxidation of LiP (the active component of cosmetic lightening cream) by hydrogen peroxide (an activator, which activates the enzyme on application on the skin) as in a typical catalytic reaction of LiP. Step 2 involves the reduction of oxidized LiP by a molecule of veratryl alcohol (VA), a substrate specific for LiP, leading to the production of a veratryl alcohol radical ($VA^{\bullet+}$) which in turn mediates the oxidation of melanin on the skin in step 3. In step 4, LiP is inactivated by change in pH which occurs as a result of application of the enzyme on the skin, thereby becoming a simple glycoprotein which is subsequently hydrolysed into amino acids by proteases and other glycosidases naturally present in the skin in the last step (step 5).

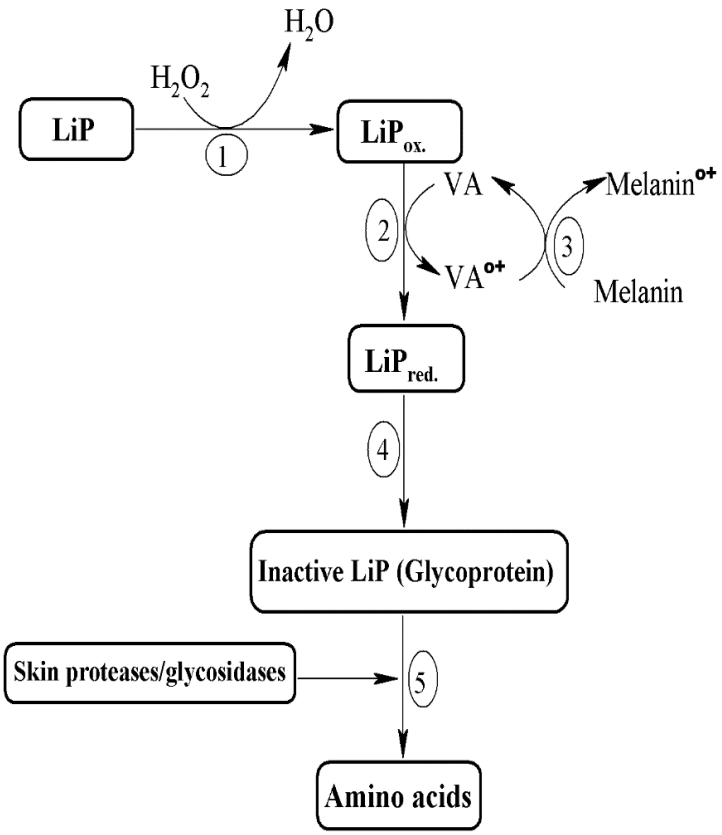

Fig. 2.5. Mechanism of action of lignin peroxidase as cosmetic lightening agent. Step 1; oxidation of LiP by hydrogen peroxide, Step 2; reduction of oxidized LiP by one molecule of veratryl alcohol (VA), Step 3; oxidation of melanin, Step 4; inactivation of LiP by change in pH to become a simple glycoprotein, Step 5; hydrolysis of glycoprotein into amino acids by proteases and other glycosidases naturally present in the skin (Falade *et al.*, 2017a).

2.6.6. Removal of endocrine disrupting chemicals in wastewater

The occurrence of endocrine disrupting chemicals (EDC) as organic contaminants in surface waters including rivers has become an environmental and public health concern due to their potential health effects and ecological risks (Boxall *et al.*, 2012; You *et al.*, 2015).

There are recent reports of occurrence of organic micropollutants (Ratola *et al.*, 2012) and EDC in wastewater and the receiving waterbodies around the world (Olujimi *et al.*, 2012; Barber *et al.*, 2015; Komesli *et al.*, 2015; Noutsopoulos *et al.*, 2015; Salgueiro-González *et al.*, 2015; Vajda *et al.*, 2015). The presence of EDC in freshwaters has been attributed to indiscriminate and direct

municipal wastewater effluent discharge from wastewater treatment plants (You *et al.,* 2015), as most treatment plants do not possess the technology for complete removal of EDC. Other sources of EDC include different anthropogenic activities, agricultural activities, pharmaceuticals, personal care products, spraying of pesticides and herbicides, artificial chemicals, use of air fresheners, sunscreens, beverage cans etc.

EDC possess inherent ability to alter the endocrine system thereby interfering with the organism's hormonal coordination, consequently affecting the development, behaviour and reproductive system of the organisms (Caliman and Gavrilescu, 2009; You *et al.,* 2015). Major classes of EDC include but not limited to pharmaceuticals and personal care products (PPCPs), phthalates, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), alkylphenols (APs), alkylphenol ethoxylates (APEs), pesticides including dichlorodiphenyltrichloroethane (DDT) and plastic additives such as Bisphenol A (Annamalai and Namasivayam, 2015). The high environmental and health risk posed by exposure of human to EDC and the inefficiency of the conventional treatment approaches for complete removal of EDC in wastewater, as well as some challenges that characterized the conventional treatment processes have led to an increased interest in the exploration of alternative treatment processes for EDC removal in wastewater.

Enzymatic treatment process for EDC removal based on the use of ligninolytic oxidative enzymes (Table 2.2) has recently attracted interest as an environmental friendly alternative. The potential of some enzymes including LiP, MnP, VP, laccases, tyrosinase and lipase for efficient removal of EDC in water has recently been reported (Wen *et al.*, 2009, 2010; Zhang and Geissen, 2010; Diano and Mita, 2011; Taboada-Puig *et al.,* 2011; Touahar *et al.,* 2014; Ramirez-Cavazos *et al.,* 2014; Garcia-Morales *et al.,* 2015). However, among the studied enzymes, VP seems to be the most promising, given its peculiar attribute of hybrid molecular architecture and its ability to oxidize both high and low redox potential substrates. This assertion is justified by Rodriguez *et al.* (2004) in their study of the role of ligninolytic enzymes (laccase and VP) in the degradation of phenolic and non-phenolic aromatic pollutants, 2,4-Dichlorophenol (2,4-DCP) and Benzo[a] pyrene (B[a]P) by four *Pleurotus* species (*Pleurotus eryngii*, *Pleurotus ostreatus*, *Pleurotus pulmonarius* and *Pleurotus sajor-caju*) wherein it was reported that versatile peroxidase oxidized 2,4-DCP faster than laccase with 100 % transformation after 1 h and 60 % transformation after 6hr respectively.

The same trend was observed in the oxidation of B[a]P as VP showed 40% transformation of B[a]P after 6 h while laccase exhibited 25% transformation in the presence of a mediator after 8 h.

Davila-Vazquez *et al.* (2005) studied the potential of purified VP from *Bjerkandera adusta* UAMH 8258 to transform several pesticides such as pentachlorophenol (PCP). The study showed that VP was able to transform dichlorophen, bromoxynil and PCP in the presence and absence of Manganese (II) and further suggested its potential use in the enzymatic transformation of halogenated toxic compounds.

Taboada-Puig *et al.* (2011) produced a combined cross-linked enzyme aggregate from versatile peroxidase and glucose oxidase (combined CLEAs) and investigated its ability to eliminate the following endocrine disruptors: bisphenol A, nonylphenol, triclosan, 17α-ethinylestradiol and 17β-estradiol. It was reported that co-aggregation of versatile peroxidase with glucose oxidase resulted in an increased activity recovery of 89 % from the initial activity of 67 % and an increased stability of VP against H_2O_2. The combined CLEAs were able to remove all the endocrine disrupting chemicals except triclosan while the removal of their estrogenic activity was more than 55 % for all the EDC except triclosan. The exploration of other H_2O_2–producing enzymes with more appropriate substrates in water treatment other than glucose in the case of glucose oxidase which might support the unwanted growth of microorganisms has been suggested. Adoption of this concept for other ligninolytic peroxidases for EDC removal and other applications is also desirable.

Furthermore, Touahar *et al.* (2014) investigated the ability of a combined cross-linked enzyme aggregates (combi-CLEA) {comprising laccase, versatile peroxidase and glucose oxidase} to transform a cocktail of pharmaceutically active compounds (PhACs) in a mixed solution and synthetic wastewater. The free enzymes and combi-CLEA showed the ability to efficiently transform non-steroidal anti-inflammatory drugs (acetaminophen, naproxen, mefenamic acid, diclofenac and indometacin) in a mixed solution and eliminate acetaminophen in municipal wastewater. However, combi-CLEA exhibited a more improved removal efficiency. The study also demonstrated that versatile peroxidase had a wider removal spectrum than laccase.

Toboada-Puig *et al.* (2015) in a recent study, utilized the oxidant, Mn^{3+}-malonate generated by VP in a two-stage (TS) system for continuous removal of the following endocrine disrupting compounds: bisphenol A, triclosan, estrone, 17β-estradiol and 17α-ethinylestradiol from synthetic

and real wastewaters at degradation rates ranging from 28 to 58 µg/L.min, with little enzyme inactivation observed. Interestingly, a 14-fold increase in the EDC removal efficiency of VP in a TS system was observed when compared with a regular enzymatic membrane reactor (EMR) system. Also, some of the operational challenges encountered during EDC removal in an EMR system were prevented, as the TS system was able to separate the complex formation stage from the contaminant oxidation stage. It is noteworthy that VP in a TS enzymatic system exhibited 100% removal efficiency for all the EDC studied, therefore demonstrating the practicability of this approach for removing endocrine disrupting chemicals at both high and environmental concentrations.

Table 2.2. EDC removal by enzymatic approach

Water Source	**Classes of EDC**	**Percentage Removal (%)**	**EDC Removal Approach**	**References**
Wastewater	Bisphenol A (BPA) Triclosan Estrone (E1) 17β-estradiol (E2) 17α-ethinylestradiol (EE2)	100	Versatile peroxidase using two stage system (TSS)	Taboada-Puig *et al.* (2015).
Synthetic and ground water	Bisphenol A 4-nonylphenol 17α-ethynlestradiol Triclosan	89 93 100 90	Free laccase cocktail	Garcia-Morales *et al.* (2015)
Wastewater	Acetaminophen Mefenamic acid Carbamazepine	93	Cross-linked laccase aggregates and polysulfone hollow fibre microfiltre membrane	Ba *et al.* (2014)
Wastewater	Estrone 17β-estradiol (E2) 17α-ethinylestradiol (EE2)	83.6 94 93.6	Laccase using enzymatic membrane reactor (EMR)	Lloret *et al.* (2013)

Aqueous system	Estrone 17β-estradiol (E2) 17α-ethinylestradiol (EE2)	65 80 80	Immobilized Laccase in a packed-bed reactor	Lloret *et al.* (2012)
Aqueous system	Diclofenac and estrogen hormones Sulfamethoxazole and Naproxen	100 80	Versatile peroxidase	Eibes *et al.* (2011)
Wastewater	Bisphenol A, B, F Bisphenol A, B, F	100 92, 93, 94	Immobilized laccase Immobilized tyrosinase	Diano and Mita (2011)
Wastewater	Estrone Estriol 17β-estradiol (E2) 17α-ethinylestradiol	100	Horseradish peroxidase and laccase	Auriol *et al.* (2008)
Simulated wastewater	Nonylphenol Bisphenol A Triclosan	100 100 65	Laccase	Cabana *et al.* (2007b)
Simulated wastewater	Nonylphenol Bisphenol A Triclosan	100	Immobilized laccase in fluidized bed reactor	Cabana *et al.* (2007a)
Aqueous system	Natural steroidal hormone, estrone	98	Manganese peroxidase and laccase	Tamagawa *et al.* (2006)
Aqueous system	Genistein	93	Manganese peroxidase and laccase	Tamagawa *et al.* (2005)
Aqueous system	Bisphenol A Nonylphenol	100	Manganese peroxidase and laccase-1-hydroxybenzotriazole (laccase-HBT) system	Tsutsumi *et al.* (2001)

2.6.6.1. Proposed mechanisms for EDC removal by VP

- **Mn^{2+} Oxidation**

  In this mechanism, VP employs the MnP reaction pathway for the removal of EDC in wastewater. This mechanism is proposed as the most appropriate for the removal of phenolic EDC including Bisphenol A and Nonylphenol. Mn^{2+} Oxidation involves the following steps:

  Step 1: Enzyme Activation: This step involves oxidation of VP by hydrogen peroxide (H_2O_2) as in a typical peroxidase catalytic reaction (Fig. 2.3).

  Step 2: Reduction of VP: The oxidized VP is reduced by a molecule of Manganese II Sulphate ($MnSO_4$), a typical substrate for manganese peroxidase, leading to the production of $Mn^{3+.}$

  Step 3: Formation of oxidizing complex: The Mn^{3+} produced in step 2 is stabilized by forming a complex with a dicarboxylic organic acid such as malonate, oxalate and tartrate which is present in the reaction mixture as buffer (sodium malonate, sodium oxalate or sodium tartrate).

  Step 4: Degradation of EDC by Mn^{3+}-complex: Endocrine disrupting chemicals and other emerging organic micropollutants are degraded by Mn^{3+} in a displacement reaction.

- **Long Range Electron Transfer (LRET)**

  LRET mechanism of VP is hereby proposed for the removal of EDC in wastewater. This mechanism involves electron transfer from the exposed catalytic tryptophan, Trp^{164} to the heme thereby generating a tryptophanyl radical on the surface of versatile peroxidase. The tryptophanyl radical subsequently attacks the endocrine disrupting chemicals and other emerging pollutants. LRET mechanism takes care of non-phenolic EDC and high redox potential aromatic compounds. A scheme of VP catalytic reactions for EDC removal is presented in Fig. 2.3.

2.6.6.2. Proposed scheme of wastewater treatment process for EDC removal by VP

The present wastewater treatment technology involves three different stages including primary treatment, secondary treatment and tertiary treatment. Each stage has specific units for specified treatment (Fig. 2.6). However, no specific unit is designed to remove endocrine disrupting chemicals during wastewater treatment. Therefore, a scheme of wastewater treatment process that includes a specific unit for the removal of endocrine disrupting chemicals at the tertiary treatment stage is proposed (Fig. 2.6). In most cases, the tertiary wastewater treatment stage involves only nutrient removal after which the effluent is discharged into the receiving water bodies. There are several reports of occurrence of endocrine disrupting chemicals in wastewater treatment plant effluents (Ra *et al.*, 2011; Ifelebuegu, 2011; Martin *et al.*, 2012; Huang *et al.*, 2014; Pessoa *et al.*, 2014). Furthermore, Zhang *et al.* (2016a) gave a detailed report of the occurrence and distribution of endocrine disrupting chemicals in wastewater treatment plants worldwide.

Before the final effluent is discharged into the aquatic environment, the effluent from the nutrient removal unit is passed through an enzymatic membrane reactor unit where EDC are removed by versatile peroxidase using the Mn^{2+} oxidation or LRET mechanisms under optimized conditions. The choice of mechanism is based on the type of EDC in the effluent, while Mn^{2+} oxidation is best for removal of phenolic EDC, LRET is more appropriate for removal of non-phenolic EDC.

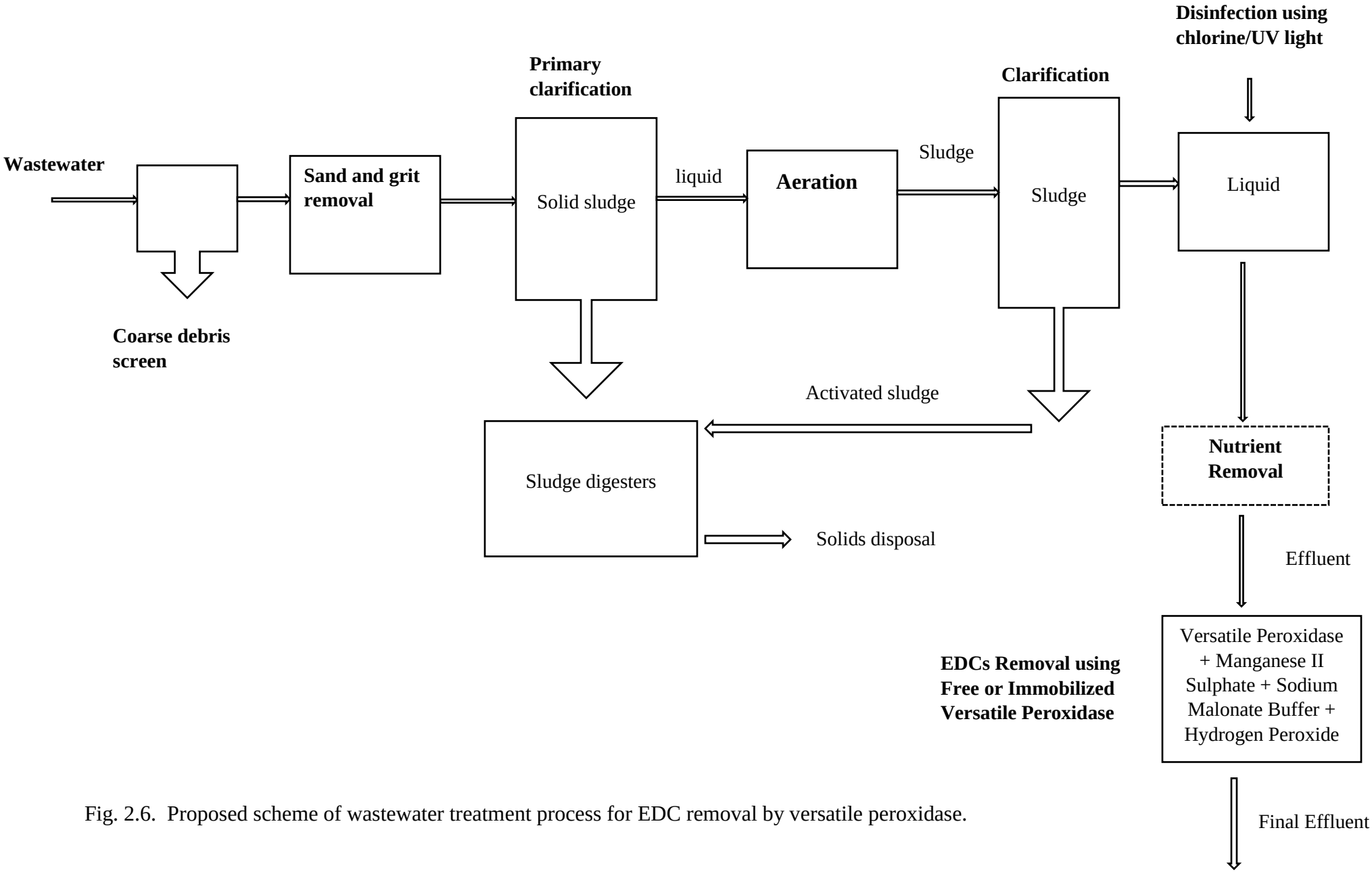

Fig. 2.6. Proposed scheme of wastewater treatment process for EDC removal by versatile peroxidase.

2.7. Peroxidase production

The major commercially available peroxidase, horseradish peroxidase (HRP) is of plant origin and cannot meet the increasing industrial demand on peroxidases. Hence, the imperativeness of searching for new and efficient sources of peroxidases.

The interest in microbial peroxidases has continued to increase as microbial enzymes seem to be more stable than enzymes of plant and animal origin (Anbu *et al.,* 2013). More so, peroxidases from plants and animals are intracellular in nature, consequently, their isolation and purification is complex and time-consuming as against extracellular peroxidase whose recovery and purification is simplified. The ability of microbes to produce extracellular enzymes has therefore led to the exploration of the microbial diversity for production of various enzymes of industrial significance.

White rot fungi have been described as the most efficient producers of lignocellulolytic enzymes such as cellulases, xylanase, laccase and peroxidases. Peroxidase production by fungi has been extensively reported (Ikehata *et al.* 2004; Urek and Pazarlioglu, 2007; Irshad and Asgher, 2011; Hariharan and Nambisan, 2013; Kong *et al.,* 2016; Zhang *et al.,* 2016b). However, peroxidase production by some bacteria, predominantly, *Streptomyces* species and very few *Bacillus* species have also been reported (Tuncer *et al.,* 2009; Nour El-Dein *et al.,* 2014; Musengi *et al.,* 2014). The fact that bacteria can easily be cultivated in a defined medium and their ability to undergo genetic manipulation make them more promising for enhanced peroxidase production.

Ligninolytic bacteria hold a great potential for peroxidase production as lignin degradation activity of these organisms is particularly accompanied by the production of extracellular LMEs, predominantly, peroxidases, as secondary metabolites which can easily be harvested and exploited for various biotechnological applications.

The increased industrial applications of peroxidases as well as their increasing market demand have necessitated the need for increased enzyme production. Several efforts have been made to increase peroxidase production by microbes. These efforts largely involved optimization of culture and nutritional conditions of microbes for peroxidase production using the conventional method or response surface methodology (RSM).

In conventional method, optimal fermentation process parameters (medium pH, incubation temperature and agitation speed), which are significant for optimum growth and metabolic

activities of an organism are determined one after the other as the continuous secretion of ligninolytic enzymes into the fermentation medium has been associated with bacterial growth (McCarthy, 1987; Niladevi and Prema, 2008; Musengi *et al.* 2014). Besides, another important element that affects the growth of an organism is the nutritional composition of the cultivation medium including carbon source and nitrogen source. Carbon source selection is an important factor in enzyme production (Brito-Cunha *et al.,* 2013) as it serves as the source of energy for the organism. Most bacteria use simple sugars such as glucose, fructose, sucrose, lactose etc as sources of carbon in a minimal salt medium but this might not be the most appropriate source of carbon for enhanced peroxidase production as LMEs are inducible enzymes whose production requires the presence of an inducer as the sole carbon source in the fermentation medium. Therefore, lignin and lignin-allied compounds are the most appropriate sources of carbon that may enhance the production of LMEs. However, considering the cost implication of synthetic lignin, there is need to explore cheap and alternative sources of lignin for fermentation, and lignocellulosic biomass may serve this purpose. Abundance, availability and renewable nature bestow lignocellulosic biomass the status of near perfect candidature of a cheap carbon source. Consequently, a variety of lignocellulosic materials have been valorized for different enzyme production processes (Reddy *et al.,* 2003b; Kang *et al.,* 2004; Asgher *et al.,* 2012a, b; Knezevic *et al.,* 2013), and a conspectus of some of these processes has been articulated in Table 2.3.

The choice of nitrogen sources is another important factor that affects microbial growth and subsequent enzyme production. Consequently, the effects of form and concentration of nitrogen sources on production of LMEs have been studied extensively (Kachlishvili *et al.,* 2005; Mikiashvili *et al.,* 2006; Stajic´ *et al.,* 2006; Prasher and Chauhan, 2015), but there are discrepancies in the findings from these studies. Some studies have reported increased production of LMEs with adequate amount of nitrogen (Kaal *et al.,* 1995). On the other, ligninolytic enzyme production was improved under limited nitrogen (Mester and Field, 1997; Gianfreda *et al.,* 1999; Galhaup *et al.,* 2002). More so, increased concentration of nitrogen may also repress the production of ligninolytic enzymes. The effect of different forms of nitrogen (organic and inorganic) seems to be different with organisms. Mikiashvili *et al.* (2006) reported that inorganic nitrogen sources decreased the production of LMEs including peroxidase whereas in a study by Prasher and Chauhan (2015), inorganic nitrogen sources supported maximum enzyme activity as organic nitrogen sources repressed ligninolytic enzyme activities. Considering the peculiarity of

LMEs, care must be taken to select the appropriate form of nitrogen that would enhance the enzyme production.

Some of the research efforts toward improving peroxidase production by bacteria using the conventional approach of "one variable at a time" include the work of Nour El-Dein *et al.* (2014), where the optimal conditions for peroxidase production by *Streptomyces* sp. K37 were determined. The study reported pH 7, incubation temperature of 40^{o}C and agitation speed of 150 rpm as the culture conditions that supported maximum peroxidase production by *Streptomyces* sp. K37 while oat spelt xylan (1% w/v) and yeast extract (0.2% w/v) were used as sources of carbon and nitrogen respectively. It is noteworthy that increased concentration of yeast extract above 0.2% w/v significantly decrease peroxidase production by the bacteria.

Musengi *et al.* (2014) also optimized peroxidase production by *Streptomyces* sp. BSII#1, up to 31 culture volumes. The authors reported 1.30 ± 0.04 U mL^{-1} in 10 mL culture volume as the maximum peroxidase production at pH 8, temperature of 37 ^{o}C and 160 rpm agitation speed in the presence of 0.1 $mmoL^{-1}$ veratryl alcohol. The study reported wheat bran (1.5%) as the only natural lignocellulosic compound that improved peroxidase production by *Streptomyces* sp. BSII#1. The optimum peroxidase production achieved in the study was claimed to be higher than what was reported by previous related studies (Musengi *et al.*, 2014).

Furthermore, RSM has also been employed for optimization of ligninolytic enzyme production. This approach involves the use of a Plackett-Burman design to identify the variables that have significant effects on enzyme production during the initial screening. The identified variables are then selected for further optimization using an appropriate design such as central composite design (CCD), Box-Behnken Design (BBD) etc. Bonugli-Santos *et al.* (2010) employed 2^2 statistical experimental design and CCD for peroxidase production by some marine-fungi. Also, Yasmeen *et al.* (2013) were able to enhance peroxidase production by *Schyzophyllum commune* and *Gonaderma lucidum* using RSM with CCD in a solid-state fermentation (SSF) using corn stover as a substrate.

Table 2.3. Valorization of some lignocellulosic biomass for ligninolytic and cellulolytic enzyme production.

Lignocellulosic Biomass	**Microorganism**	**Enzyme Produced**	**References**
Rice straw	*P. chrysosporium;* *T. versicolor*; *Trichoderma reesei*; *Aspergillus niger* KK2.	LiP, MnP, Laccase, Cellulases, Hemicellulases.	Kang *et al.* (2004), Iqbal *et al.* (2011), Asgher *et al.* (2011), Saratale *et al.* (2014).
Sugarcane bagasse	*Thermoascus aurantiacus*; *Bacillus circulans*; *Trametes villosa.*	Xylanase and MnP	Milagres *et al.* (2004), Bocchini *et al.* (2005), Silva *et al.* (2014).
Wheat straw	*Phlebia radiata*; *Trichoderma viride*; *Trametes suaveolens*;	LiP, MnP, Laccase, Cellulase,	Vares *et al.* (1995), Iqbal *et al.* (2011), Knezevic *et al.* (2013),
	Raoultella ornithinolytica OKOH- 1; *Ensifer adhaerens* NWODO-2.	Peroxidase.	This study.
Banana waste	*P. ostreatus*; *P. sajor-caju*; *Schizophyllum commune* IBL-06	LiP, MnP, Laccase, Xylanase, Endoglucanase, Exoglucanase.	Reddy *et al.* (2003b), Irshad and Asgher (2011), Asgher *et al.* (2012c).
Corn cobs	*Trametes versicolor*	LiP, MnP, Laccase.	Asgher *et al.* (2012a, b)
Sawdust	*Trametes suaveolens;* *Raoultella ornithinolytica* OKOH- 1; *Ensifer adhaerens* NWODO-2.	MnP, Laccase, Peroxidase.	Knezevic *et al.* (2013). This study
Pea pods	*Aspergillus niger* HN-1	Filter Paper Cellulase (FPase) and β- glucosidase (BGL).	Sharma *et al.* (2015).
Corn stover	*Raoultella ornithinolytica* OKOH- 1; *Ensifer adhaerens* NWODO-2.	Peroxidase	This study.

LiP: Lignin Peroxidase, MnP: Manganese Peroxidase.

2.7.1. Gene detection: towards molecular optimization

Molecular optimization and genetic engineering approach seems to be the best option in solving the problem of low enzyme production yields, which is one of the major obstacles in the biotechnological applications of enzymes. Hence, the imperativeness of detecting the gene encoding the expression of the enzyme of interest in an organism as well as characterizing the biosynthetic pathway which could be engineered for large scale production. Identifying the genes encoding microbial enzymes may enhance their expression through metabolic engineering approaches such as "gene disruption" and "overexpression" (Tamano, 2014) employing genetic modification tools (Andrio and Demain, 2010). In case the genes involved in the biosynthesis of a metabolite are not known, the production yield can be improved by random chromosomal mutation of the producing organism through ultraviolet irradiation or introduction of mutagens (Andiro and Demain, 2006; Tamano, 2014).

The genes encoding microbial enzymes can possibly be identified using the N-terminal amino acid sequences and molecular weights of purified enzymes as well as the genomic data of the synthesizing microbes (Tamano, 2014). Consequently, the overexpression of the identified gene could then be induced in the original producing organism or another host to enhance the enzyme production. This approach has been employed to enhance the production of some enzymes and metabolites as reported in literature. For instance, overexpression of the gene encoding phosphoenolpyruvate carboxylase (*ppc*) in *E. coli* led to a 3.5-fold increase in succinic acid production (Millard *et al.*, 1996). Sekhon *et al.* (2011) were able to increase biosurfactant production by cloning biosurfactant genes from a Bacillus species in *E. coli.* Likewise, Velez *et al.* (2013) enhanced lipase production in *E. coli* through recombinant DNA technology. Furthermore, fatty acids production by *Aspergillus oryzae* was increased by 2.8-fold when the fatty acid synthase genes were overexpressed (Tamano *et al.*, 2013). It is therefore clear that this approach could also be employed to enhance the production of peroxidases. Thus, the need to detect the genes encoding peroxidases in the producing organisms.

2.8. Conclusion

The prospects of peroxidase in bio refinery, bioremediation, cosmetology and dermatology amongst other endeavours of human activities cannot be over emphasized, its potential as a suitable alternative to hydroquinone in the development of skin-lightening cream and treatment of

hyperpigmentation as well as its potential in the removal of EDCs in wastewater have been properly synopsized in this review. Besides, the prospects of other ligninolytic enzymes systems, not yet known, abound in all the aforementioned industries and beyond. In view of the articulated importance and prospective applications of peroxidase, the exploration of the underexplored microbial diversity for novel peroxidase with enhanced capabilities in solving problems as envisaged in this review becomes pertinent. Besides the need of peroxidase for remediation of toxic phenolics amongst others in the environment and its application in cosmetics and wastewater treatment, jobs and economy will be boosted thus, improving upon the social standing of any community with significant inroad into this lucrative bio-economic sector.

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CHAPTER THREE

Peroxidase production and ligninolytic potentials of freshwater bacteria *Raoultella ornithinolytica* and *Ensifer adhaerens*

(*Published in Biotechnology Reports*)

Abstract

Interest in novel ligninolytic bacteria has remained topical due to, in part, the maneuverability of the bacterial genome. Conversely, the fungal genome lacks the dexterity for similar maneuverability thus, posing challenges in the fungal enzyme yield optimization process. Some impact of this situation includes the inability to commercialize the bio-catalytic process of lignin degradation by fungi. Consequently, this study assessed some freshwater bacteria isolates for ligninolytic and peroxidase properties through the utilization and degradation of model lignin compounds (guaiacol and veratryl alcohol) and the decolourization of selected ligninolytic indicator dyes; Azure B (AZB), Remazol Brilliant Blue R (RBBR) and Congo Red (CR). Bacterial strains with appreciable ligninolytic and peroxidase production potentials were identified through 16S rDNA sequence analysis and the nucleotide sequences deposited in the GenBank. About 5 isolates were positive for the degradation of both guaiacol (GA) and veratryl alcohol (VA) thus, accounting for about 17% of the test isolates. Similarly, AZB, RBBR and CR were respectively decolorized by 3, 2 and 5 bacterial strains thus, accounting for 10, 7 and 17 % of the test isolates. Two of the test bacterial strains were able to decolourize AZB, RBBR and CR, respectively and these bacterial strains were identified as *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 with respective accession numbers as KX640917 and KX640918. Upon quantitation of the peroxidase activities; 5.25 U mL^{-1} was recorded against *Raoultella ornithinolytica* OKOH-1 and 5.83 U mL^{-1} against *Ensifer adhaerens* NWODO-2. The ligninolytic and dye decolourization properties of *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 marks for novelty particularly, as dyes with arene substituents were decolourized. Consequently, the potentials for the industrial applicability of these test bacterial strains abound as there is a dearth of information on organisms with such potentials.

Keywords: Dye decolourization, ligninolytic bacteria, lignin degradation, peroxidase, proteobacteria.

Chemical compounds studied in this article:

Azure B (PubChem CID: 68275)

Congo red (PubChem CID: 11313)

Guaiacol (PubChem CID: 460)

Pyrogallol (PubChem CID: 1057)

RBBR, Remazol Brilliant Blue R (PubChem CID: 17409)

Veratryl alcohol (PubChem CID: 7118).

3.1. Introduction

Lignin; the aromatic, non-carbohydrate, component of lignocellulose is recalcitrant to degradation. Thus, effective degradation of lignin is of prime importance to the industrial sectors utilizing lignocellulose as raw materials for various value-added products (de Gonzalo *et al.,* 2016). More so, the recalcitrance of lignin to degradation constitutes an undesirable barrier to the efficient and optimum utilization of the abundant lignocellulosic materials. On the same note, the large amount of lignin generated during industrial production of ethanol, pulp and paper making processes, accumulates and, thus, constitutes serious environmental challenge hence, the need for effective and eco-friendly lignin degradation techniques (Falade *et al.,* 2017).

The biological means of lignin degradation involves microbial or/and microbial enzymes degradative activities. This technique is advocated over the physical and chemical methods which are generally expensive and saddled with lots of other limitations (Chaturvedi and Verma, 2013; Huang *et al.,* 2013).

Fungal degradation of lignin, particularly, the white-rot basidiomycetes have been studied extensively (Ruttimann-Johnson *et al.,* 1993; Martinez *et al.,* 2004; Wan and Li, 2012) and, white-rot fungi have been reported as the most effective microbial lignin-degrader. Effectiveness in lignin degradation has been attributed to some extracellular enzymes produced by the white-rot fungi (Abdel-Hamid *et al.,* 2013). These extracellular enzymes include laccases (EC 1.10.3.2), some heme-peroxidases such as lignin peroxidase (EC 1.11.1.14), manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and dye-decolourizing peroxidase (EC 1.11.1.19). Nonetheless, industrialization of white-rot fungal bio-catalytic/extracellular enzyme process for the de-polymerization of lignin is yet to be achieved. Perhaps, the insufficiency in the maneuverability of the white-rot fungal genome for optimum extracellular enzyme yield, as a function of production cost to commercial value quotient may have constituted an important factor impeding industrialization of the process (Bugg *et al.,* 2011; Ausec *et al.,* 2011).

Bacteria, on the other hand, hold very strong potential considering their striking resilience in diverse environments and, as well, their biotechnological significance following, faster growth rate and high dexterity in genome maneuverability (Ausec *et al.,* 2011; Tian *et al.,* 2016). Hence, the imperativeness in the exploration of bacteria species for lignin depolymerization potentials. Besides, the evolving significance of bacteria in the degradation of lignin has been severally

documented (Bugg *et al.,* 2011). Bacteria species classed into the actinomycetes, α-proteobacteria and γ-proteobacteria have been reported to possess lignin degrading ability (Bugg *et al.,* 2011; Paliwal *et al.,* 2012; Furukawa *et al.,* 2014). Documented ligninolytic bacteria includes *Streptomyces viridosporus* T7A, *Rhodococcus* sp, *Nocardia autotrophica* (Zimmermann, 1990), *Microbacterium* sp, *Brucella melitensis*, *Ochrobactrum* sp, *Sphingomonas* sp (Wenzel *et al.*, 2002), *Streptomyces coelicolor, Arthrobacter globiformis, Rhodococcus jostii RHA1, Pseudomonas putida mt-2* (Ahmad *et al.*, 2010), *Serratia* sp. JHT01, *Serratia liquefacien* PT01, *Pseudomonas chlororaphis* PT02, *Stenotrophomonas maltophilia* PT03 and *Mesorhizobium* sp. PT04 (Tian *et al.,* 2016).

Ligninolytic bacteria similarly produce extracellular oxidative enzymes including peroxidases which have been implicated in lignin degradation. Besides the association of these extracellular peroxidases in lignin degradation, they have applications in the removal of phenolic pollutants (Cheng *et al.,* 2006), synthetic dye decolourization (Singh *et al.*, 2014), and the synthesis of natural aromatic flavours (Santos *et al.,* 2004; Barbosa *et al.,* 2008). Other applications have likewise included deodourization of manure (Govere *et al.,* 2007), applications in peroxidase biosensors (Jia *et al.,* 2002), analysis and diagnostic kits (Agostini *et al.,* 2002) and development of skin lightening agents (Mauricio *et al.,* 2011; Draelos, 2015). Given, the diverse applications of peroxidases in different industrial sectors, the exploration of bacteria species with novel ligninolytic abilities and high potentials for peroxidase production is of prime importance. Consequently, the reported study evaluated bacterial isolates from freshwater milieu of the Raymond Mhlaba Municipality, Eastern Cape, South Africa for peroxidase production potentials and ligninolytic activities.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Hydrogen peroxide, Pyrogallol, Magnesium sulphate, Manganese II sulphate, Sodium chloride, Ammonium nitrate, Potassium dihydrogen phosphate, Dipotassium hydrogen phosphate, Nystatin, Nalidixic acid, yeast extract, Agar bacteriological, Azure B, Remazol Brilliant Blue R and Congo red were sourced from Merck KGaA, Darmstadt, Germany while Guaiacol, Veratryl alcohol and Kraft lignin were sourced from Sigma-Aldrich, South Africa. Unless stated otherwise, all other chemicals are of analytical grade while the water was glass distilled.

3.2.2. Sample collection and sampling site

Water samples and sediments (Table 3.1) were collected from Tyhume river courses in Raymond Mhlaba Municipality, Eastern Cape, South Africa and transported on ice to Applied and Environmental Microbiology Research Group (AEMREG) Laboratory, University of Fort Hare, Alice, South Africa for analysis.

Tyhume River is situated in the Raymond Mhlaba Municipality of the Eastern Cape Province, South Africa. It originates from the Amathole mountains in Hogsback and flows through the lower coastal escarpment down to Alice, a small town with several suburbs. Sample collection was on the courses of Tyhume River, with reduced anthropogenic activities, in Alice.

3.2.3. Isolation and preliminary screening of ligninolytic bacteria

Ligninolytic bacteria were isolated by enrichment method as described by Sasikumar *et al.* (2014) with minor modifications. Briefly, a phosphate buffered (pH 7) minimal salts medium (MSM) containing 4.55 g/L K_2HPO_4, 0.53 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ and 5 g/L NH_4NO_3 (Chandra *et al.,* 2008) was supplemented with 1 g/L of commercially available kraft lignin (Sigma-Aldrich, South Africa) and 0.1 g/L yeast extract (MSM-L). The components were mixed and the resulting broth autoclaved at 121 ^oC for 15 min. Enrichment culture was performed in 250 mL Erlenmeyer flask by placing 5 g of sample in 95 mL MSM-L and the culture was incubated in an orbital shaking incubator at 30 ^oC and 140 rpm for 168 h (Sasikumar *et al.,* 2014). Enriched sample of 1 mL was transferred to 9 mL of sterile normal saline and stirred vigorously at room temperature. Using 1 mL of the liquid mixture, serial dilutions were prepared. Thereafter, 100 µL of serially diluted sample was spread on MSM-L agar plate amended with 50 mg/L nystatin (to inhibit the growth of fungi) and the plates were incubated at 30 ^oC for 168 h until colonies developed. Subsequently, the cultures were purified and presumptive ligninolytic bacteria stored in 20 % glycerol at −80 °C for further analysis.

3.2.4. Evaluation of ligninolytic activity

The ligninolytic activities of isolates were assessed through the utilization and degradation of model lignin compounds (guaiacol and veratryl alcohol) using the modified method of Taylor *et al.* (2012). Briefly, 5 µL of standard inoculum of the bacterial suspension (O.D. 600 nm ≈ 1.0) was aseptically inoculated onto guaiacol and veratryl alcohol plates composed of the following:

K_2HPO_4 (4.55 g/L), KH_2PO_4 (0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L), guaiacol or veratryl alcohol (0.1 % v/v), and agar (15 g/L). The plates were incubated at 30 ^oC for 168 h and subsequently flooded with Gram's iodine solution to determine the zone of degradation.

3.2.5. Arene subsituent dye decolourization assay

Decolourization of selected dyes; Azure B (AZB), Remazol Brilliant Blue R (RBBR) and Congo Red (CR) is indicative of ligninolytic activity. Consequently, isolates with ligninolytic activity (section 2.4) were assessed following the methods of Bandounas *et al.* (2011). In brief, 5 µL of 18h culture was aseptically inoculated onto dye agar plate composed of: K_2HPO_4 (4.55 g/L), KH_2PO_4 (0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L), glycerol (40 mM), dye (100 mg/L AZB and RBBR; 50 mg/L CR), and agar (15 g/L). The plates were then incubated at 30 ^oC for 168 h and examined daily for growth and development of decolourization zones.

3.2.6. Qualitative determination of peroxidase activity

The peroxidase activity was determined qualitatively using the method proposed by Rayner and Boddy (1988) as reported by López *et al.* (2006). Briefly, isolates were inoculated in nutrient agar and incubated at 30^oC for 48 h. Thereafter, 30 µL of 0.4% (v/v) hydrogen peroxide (H_2O_2) and 1% pyrogallol in water were added to colonies. Colonies with yellow-brown colour were recorded as positive.

3.2.7. Peroxidase production and crude enzyme preparation

Peroxidase was produced in a submerged fermentation; about 2% standard inoculum of bacterial suspension in normal saline (O.D. 600 nm ≈ 1.0) was aseptically inoculated in 250 mL Erlenmeyer flasks containing 100 mL of the fermentation media composed of the following: K_2HPO_4 (4.55 g/L), KH_2PO_4 (0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L) and 0.1 % w/v kraft lignin (Sigma-Aldrich, South Africa) at pH 7. The culture was subsequently incubated in an orbital shaking incubator at 30 ^oC and 140 rpm for 48 h, the period initially used for the qualitative determination of peroxidase activity. Thereafter, cultures were aseptically withdrawn and centrifuged at 15000 rpm for 10 min at 4 ^oC using benchtop centrifuge (SIGMA 1-14K) to remove the cells. Recovered supernatant was subsequently utilized as crude enzyme for peroxidase assays.

3.2.8. Peroxidase activity assay

Peroxidase activity was measured by the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin as described by Chance and Maehly (1955) with slight modification. Reaction mixture (350 µL) contained 5 % w/v pyrogallol in 100 mM potassium phosphate buffer (pH 6) and 25 µL of culture supernatant. The reaction mixture without the crude enzyme served as the blank. The reaction was activated through the addition of 0.5 % v/v hydrogen peroxide (30% w/w) and the linear increase in absorbance at 420 nm was monitored per 34 s at 25 ^{o}C using SynergyMx 96-well microtitre plate reader (BioTeK Instruments). The peroxidase activity was subsequently calculated (Appendix D).

3.2.9. Bacterial identification using 16S rDNA sequence analysis

Bacterial isolates with the best ligninolytic and peroxidase production potentials were characterized by 16S rDNA sequence analysis. Briefly, bacterial DNA was extracted using the ZR Fungal/Bacterial DNA Kit^{TM} (Zymo Research). Thereafter the 16S target region was amplified using DreamTaq^{TM} DNA polymerase (Thermo Scientific^{TM}) and the following universal primers: 16S-27F (5' AGAGTTTGATCCTGGCTCAG 3') and 16S-1492R (5' CGGTTACCTTGTTACGACTT 3'). Subsequently, the polymerase chain reaction (PCR) products were gel extracted using Zymoclean^{TM} Gel DNA Recovery Kit (Zymo Research), and sequenced in the forward and reverse directions on the ABI PRISM^{TM} 3500xl Genetic Analyser. The sequencing products which were further purified by ZR-96 DNA Sequencing Clean-up Kit^{TM} (Zymo Research) were analysed using CLC Main Workbench 7 followed by a BLAST search in National Centre for Biotechnology Information (NCBI) (Altschul *et al.,* 1997). Subsequently, the phylogenetic tree showing the evolutionary relationships among selected ligninolytic bacteria available at the NCBI database was constructed by neighbour-joining method using Molecular Evolutionary Genetics Analysis software, version 7.0.21 (Kumar *et al.,* 2016). Similarly, the 16S rRNA gene sequences of the isolates were deposited in the NCBI GenBank as *Raoultella ornithinolytica* strain OKOH-1 (accession number KX640917) and *Ensifer adhaerens* strain NWODO-2 (accession number KX640918), respectively.

3.2.10. Data analysis

Results of replicates were pooled and expressed as mean ± standard deviation (STD) using Microsoft Excel Spreadsheet. Data were subsequently subjected to one-way analysis of variance (ANOVA) using GraphPad Prism 7 and the least significant difference was carried out (Zar *et al.*, 2010). Significance was accepted at $P \leq 0.05$.

3. 3. Results and discussion

3.3.1. Ligninolytic bacteria isolation and identification

A total of thirty (30) potential ligninolytic bacteria were isolated from the samples collected from the Tyhume River (Table 3.1) and, the 16S rDNA sequence analysis of the two bacteria strains with the best ligninolytic and peroxidase production potentials revealed $T1CS3^{D}$ as having 99% similarity to *Raoultella ornithinolytica* strain G.W-CD.10 (KP418804) while $T2BW3^{1}$ had 99% similarity to *Ensifer adhaerens* strain S4-6 (KY496256). The respective nucleotide sequences of the organisms were deposited in a GenBank as *Raoultella ornithinolytica* OKOH-1 (accession number KX640917) and *Ensifer adhaerens* NWODO-2 (accession number KX640918).

These ligninolytic bacteria are classified into the alpha-proteobacteria (*Ensifer adhaerens* NWODO-2) and gamma-proteobacteria (*Raoultella ornithinolytica* OKOH-1), respectively. This finding is consistent with earlier classification of ligninolytic bacteria into Actinomycetes, α-Proteobacteria and γ-Proteobacteria (Bugg *et al.,* 2011). However, the ligninolytic potential of some *Bacillus* sp. has also been reported (Bandounas *et al.*, 2011; Chang *et al.,* 2014). Some of the reported Proteobacteria with ligninolytic activity include but not limited to, *Sphingobium* sp. SYK-6 (Masai *et al.*, 2007), *Pseudomonas putida* mt-2, *Acinetobacter* sp. (Ahmad *et al.,* 2010) and *Raoultella ornithinolytica* S12 (Bao *et al.*, 2015). Genome sequencing analysis of *Raoultella ornithinolytica* strain S12 (CP010557) isolated in China has revealed many genes involved in aromatic compound degradation and other pathways implicated in lignin degradation mechanism (Bao *et al.,* 2015; Kameshwar and Qin, 2016). This further confirms the lignin degradation potential of *Raoultella ornithinolytica* OKOH-1 as claimed in this study. Furthermore, Fig. 3.1 showed the phylogenetic relationships between the ligninolytic bacteria in this study and some of those previously reported. The ligninolytic bacteria in this study (indicated with green tips) are, perhaps, more closely related.

Table 3.1. Isolated ligninolytic bacteria from freshwater milieu of Raymond Mhlaba Municipality, Eastern Cape, South Africa.

S/N	Isolate code	Isolation source	Guaiacol degradation	Veratryl Alcohol degradation
1.	T1CS3[1]	Sediment	-	-
2.	T1CS3[2]	Sediment	-	+
3.	T1CS3[3]	Sediment	-	-
4.	T1CS3[4]	Sediment	-	-
5.	T1B2S3[1]	Sediment	-	+
6.	T1B2S3[2]	Sediment	-	+
7.	T1B2S3[3]	Sediment	-	+
8.	T1B1S3[1]	Sediment	+	+
9.	T1B1S3[2]	Sediment	-	-
10.	T1B1S3[3]	Sediment	-	-
11.	T1B1S3[4]	Sediment	+	+
12.	T1B1S3[5]	Sediment	+	-
13.	T1B1W3[1]	Water	+	+
14.	T1B1W3[2]	Water	-	+
15.	T1B2W3[1]	Water	-	-
16.	T1B2W3[2]	Water	-	-
17.	T1B2W3[3]	Water	-	-
18.	T1CW3[1]	Water	-	-
19.	T1CW3[2]	Water	-	-
20.	T1CW3[3]	Water	+	-
21.	T2BS2[1]	Sediment	-	+
22.	T2BS3[1]	Sediment	-	+
23.	T2BS3[2]	Sediment	-	-
24.	T2BW3[1]	Water	+	+
25.	T2BW3[2]	Water	-	+
26.	T2BW3[3]	Water	-	-
27.	T1CS3[B]	Sediment	-	-
28.	T1CS3[C]	Sediment	-	-
29.	T1CS3[D]	Sediment	+	+
30.	T1CS3[A]	Sediment	-	-

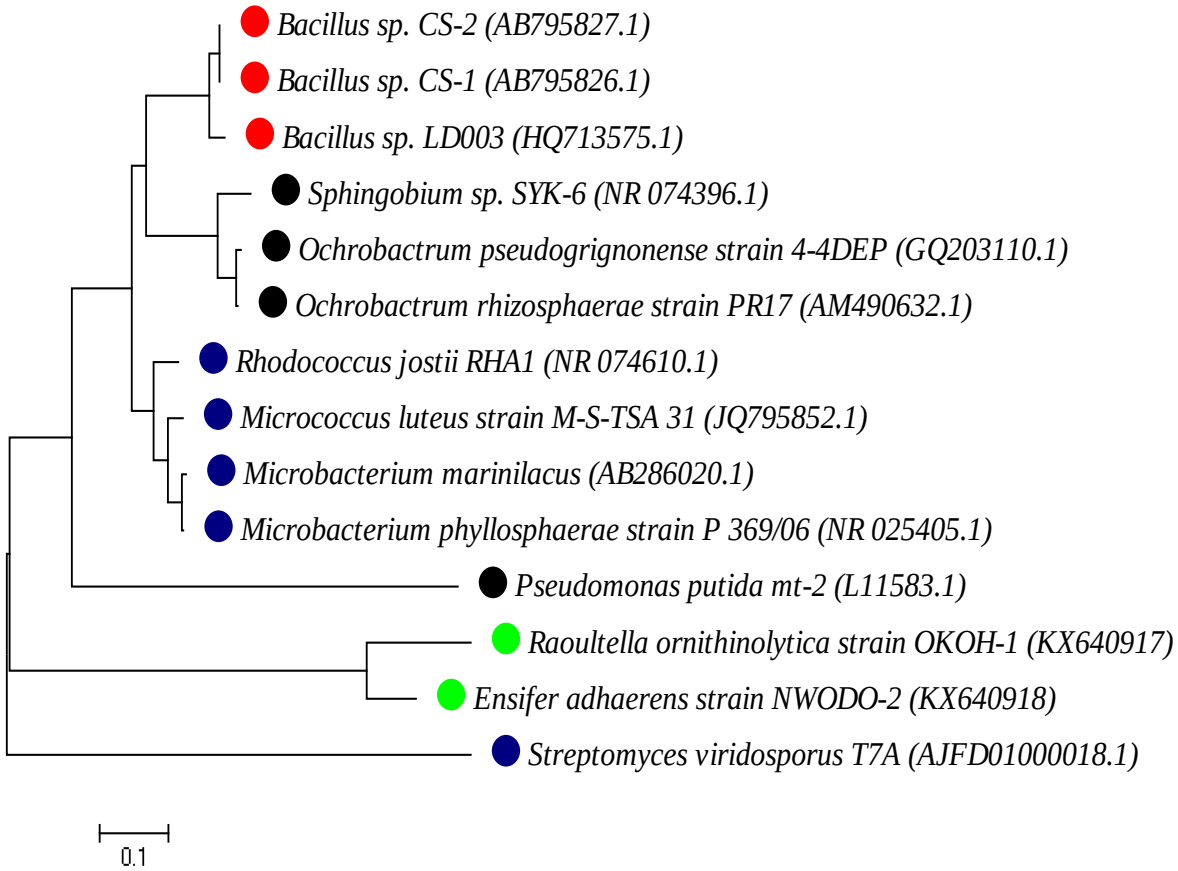

Fig. 3.1. Phylogenetic tree showing the relationship between ligninolytic bacteria in this study and some previously reported ligninolytic bacteria in the NCBI database. The tips shown in green represent the ligninolytic bacteria isolated and sequenced in this study while the tips with other colours (red, black and blue) indicate the previously reported ligninolytic bacteria. Red tips indicate Bacilli; blue tips represent Actinobacteria while the green and black tips indicate Proteobacteria.

3.3.2. Ligninolytic activities

The utilization of lignin monomers; guaiacol (2-methoxyphenol) and veratryl alcohol (3, 4-Dimethoxybenzyl alcohol), was indicative of lignin utilization and degradation potentials of the isolates. Guaiacol and veratryl alcohol utilization serves as ligninolysis indicator and as well, lignin oxidation (Bandounas *et al.*, 2011). Only 17% (5 isolates) of the test isolates were able to degrade both guaiacol (phenolic substrate) and veratryl alcohol (non-phenolic substrate). However, all the test isolates grew either on guaiacol or on veratryl alcohol. Isolates substrates utilization intensity

was determined by the zone of degradation (Table 3.2) which became visual after flooding with Grams' iodine. The reaction of hydrogen iodide (HI) with the substrates in the presence of oxygen resulted in a brown coloration of the un-degraded part of the medium while the degraded part was revealed as a clear halo zone around the colony. Isolate T2BW3[1] showed the highest halo zone on both substrates (32 mm against guaiacol and 34 mm against veratryl alcohol) while isolate T1B1W3[1] had the least (25 mm). However, about 80% of the positive isolates showed halo zones of over 25 mm.

Table 3.2. Degradation of guaiacol and veratryl alcohol by bacterial isolates

S/N	Isolate Code	Diameter of halo zone for GA (mm)	Diameter of halo zone for VA (mm)
1.	T1B1S3[1]	26.00 ± 2.00[a]	27.00 ± 1.00[a]
2.	T1B1S3[4]	27.00 ± 1.00[a]	30.00 ± 0.00[b]
3.	T1B1W3[1]	25.00 ± 3.00[a]	25.00 ± 1.00[c]
4.	T1CS3[D]	28.00 ± 0.00[a]	31.00 ± 1.00[d]
5.	T2BW3[1]	32.00 ± 0.00[b]	34.00 ± 0.00[e]

GA: Guaiacol; VA: Veratryl Alcohol. Values represent mean ± standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different ($P > 0.05$).

3.3.3. Decolourization of dyes with different arene substituents

The structural complexity of dyes is somewhat similar to those of lignin and the recalcitrance of dyes to degradation has been variously documented (Bandounas *et al.*, 2011). The enzymatic decomposition of the phenolic compounds in lignin leads to effective degradation and this is only possible due to the hydrophilic attack at the arene substituents (Srebotnik and Hammel, 2000; Christopher *et al.*, 2014). Consequently, application of such enzyme system in the decolourization of dye would only be effective if the arene substituents of the dye are susceptible to hydrolyzation (Goszczynski *et al.,* 1994).

Isolates showing ligninolytic activity on guaiacol and veratryl alcohol were evaluated for dye decolourization using Azure B (AZB), Remazol Brilliant Blue R (RBBR) and Congo Red (CR). Azure B, a thiazine dye, can only be decolourized by high redox potential agents, particularly lignin peroxidases (Archibald, 1992; Aguiar and Ferraz, 2007; Arantes and Ferreira-Milagres, 2007). On the other hand, manganese peroxidase and laccase alone cannot oxidize Azure B (Archibald, 1992; Arora and Gill, 2001). The inclusion of dyes with *ortho* and *para* arene (phenolic and non-phenolic) substituents (Fig. 3.2), was motivated by the quest to ascertain the broad spectrum of activity and specificity of the oxidative enzyme systems produced by these organisms. Congo red has two azo groups (-N=N-) which impacts the chromophore properties shown by the dye and, the azo groups are attached at the *ortho* position. Conversely, Remazol Brilliant Blue R is an anthraquinone dye with a *para* position arene substituent and this dye is recalcitrant to degradation. The carbonyl group (C=O), which constitutes the structural backbone of the dye, has been shown to impact the chromophore properties of the dye. The arene substituents position on the aromatic rings is the factor impacting degradation recalcitrant to the dyes. As such, the effective cleavage of the arene substituents at the *ortho, meta* and *para* positions marks for novelty. Thus, the natures of the enzymes produced by the organisms are, perhaps, novel and the kinetics as well as the properties shall be further investigated.

Dye decolourization (Table 3.3) showed 10% (3 bacteria strains) of the isolates as positive against AZB, 7% (2) against RBBR and 17% (5) against CR. Quite remarkable were isolates T1CS3^{D} and T2BW3^{1} which showed competence in the decolourization of dyes with the representative *ortho, meta* and *para* positions arene substituent. Perhaps, the extracellular oxidative enzymes produced by these organisms are novel or, are, known enzymes with a blend of properties including peroxidases and laccases. Nonetheless, extracellular oxidative enzyme decolourization of Azure B has been associated with lignin peroxidase (Pointing, 1991), while decolourization of azo and anthraquinone dyes are linked with the activity of DyP-type peroxidases (Sugano *et al.,* 2007; Santos *et al.,* 2014).

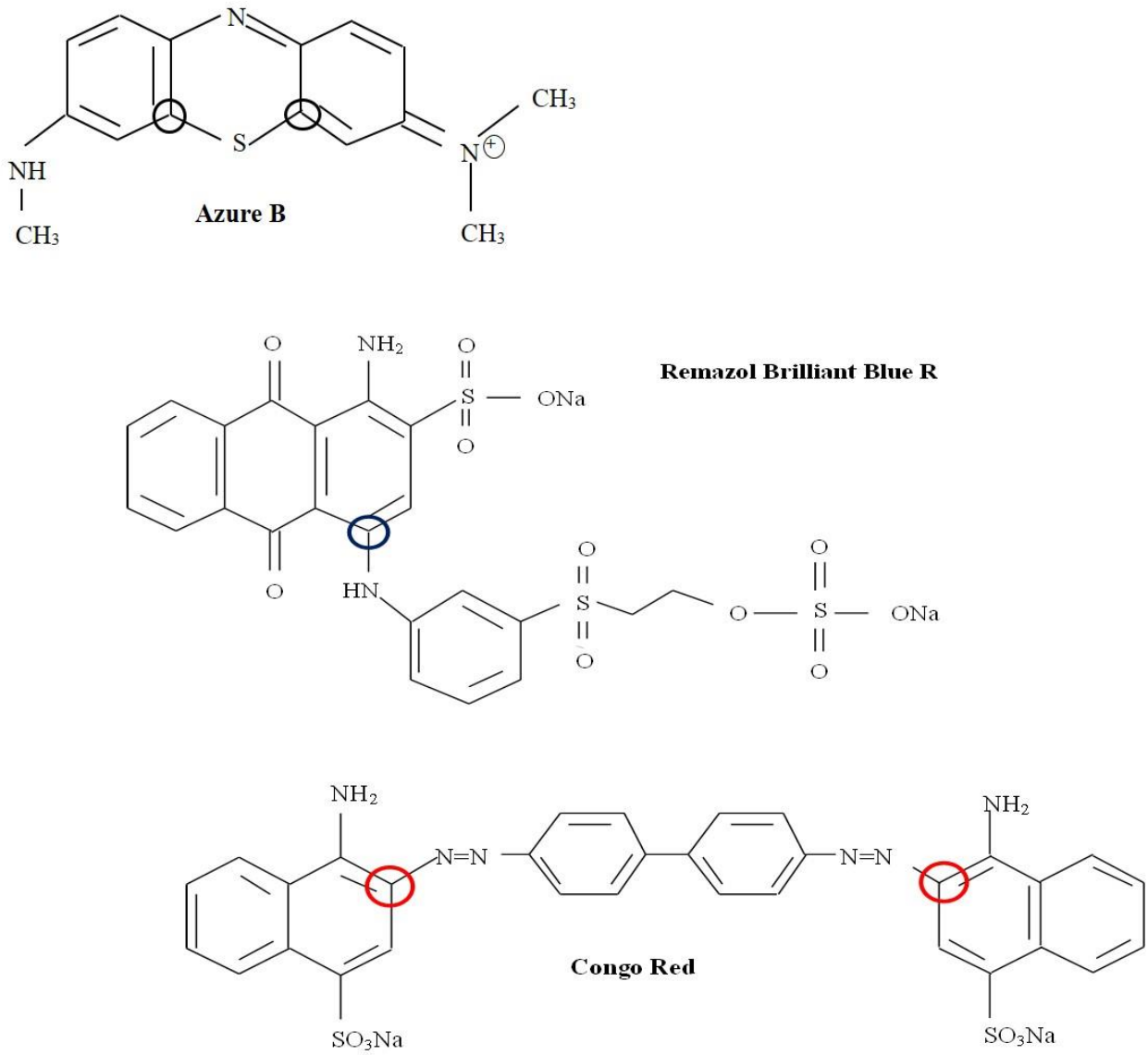

Fig. 3.2. Structures of dyes used in this study.

Ortho positions are shown in red circles, *Meta* positions in black circles while *Para* position is indicated in blue circle.

Table 3.3. Decolourization of dyes with different arene substituents

S/N	Isolate Code	AZB	RBBR	CR
1.	$T1B1S3^{1}$	-	-	+
2.	$T1B1S3^{4}$	-	-	+
3.	$T1B1W3^{1}$	+	-	+
4.	$T1CS3^{D}$	+	+	+
5.	$T2BW3^{1}$	+	+	+

+: positive; -: negative; AZB: Azure B; RBBR: Remazol Brilliant Blue R; CR: Congo Red.

3.3.4. Peroxidase activity

Isolates; $T1CS3^{D}$ and $T2BW3^{1}$, which respectively showed activity against representative dyes with *ortho*, *meta* and *para* substituents, similarly hold high potentials as peroxidase producers. These ligninolytic bacteria strains were qualitatively positive for peroxidase production (Fig. 3.3), as was reflected in the appearance of yellowish-brown colouration of the bacterial colony after interaction with 0.4% v/v hydrogen peroxide (H_2O_2) and 1% w/v pyrogallol (Lopez *et al.*, 2006).

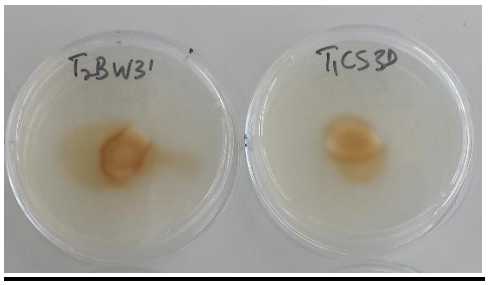

Fig. 3.3. Qualitative peroxidase activity of ligninolytic bacteria.

Upon quantitation of peroxidase production (Table 3.4), $T1CS3^{D}$ showed activity of 5.25 U mL^{-1} and $T2BW3^{1}$ showed 5.83 U mL^{-1} activity at 48 h incubation timeline. Some related investigation reported similar result however, the peroxidase activity achieved with $T1CS3^{D}$ and $T2BW3^{1}$ were significantly higher than what has been reported in previous studies; *Streptomyces* strain EC22 had an extracellular peroxidase activity of 0.27 U mL^{-1} (Mercer *et al.*, 1996) and *Streptomyces* sp. F6616 showed peroxidase activity of 0.535 U mL^{-1} (Tuncer *et al.*, 2009). The reason for the marked

difference in the peroxidase activity observed with these isolates in comparison with documented report is unclear however, it is a motivation for further investigation. Perhaps, it would be pertinent to note that the peroxidase activities shown by $T1CS3^{D}$ and $T2BW3^{1}$ are consistent with their ligninolytic activities as shown with model lignin compounds degradation (Table 3.2) and decolourization of dyes with varied arene substituent (Table 3.3). This, therefore, may be suggestive of the production of lignin modifying enzymes by the test bacterial strains including peroxidases.

Table 3.4. Evaluation of ligninolytic bacteria for peroxidase production

S/N	**Isolate code**	**Peroxidase Activity (U mL^{-1})**
1.	$T1CS3^{D}$	5.25 ± 0.00
2.	$T2BW3^{1}$	5.83 ± 0.00

Values represent mean ± standard deviation, number of replicate, n = 3.

3.4. Conclusion

The bacterial strains; $T1CS3^{D}$ and $T2BW3^{1}$, isolated from the freshwater milieu of the Raymond Mhlaba Municipality, Eastern Cape, South Africa which have shown novel ligninolytic activities were identified as *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 with KX640917 and KX640918 as respective accession numbers. These Proteobacteria strains produced extracellular enzymes with the capacity to degrade dyes with *ortho, meta* and *para* arene substituent and as such, decolourize the model dyes. Consequently, *Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2 hold high potentials for industrial applications, particularly in the dye-wastewater management process. Besides their potential industrial relevance on industrial treatment, they may serve important purpose on the pretreatment of lignocellulosic biomass, a significant step in the bioconversion of lignocellulose to ethanol. Nevertheless, further study on the mechanism(s) of action of these novel bacterial strains for lignin-degradation is imperative as this is significant to their scalability and commercial application in the future.

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CHAPTER FOUR

Peroxidase produced by ligninolytic Bacillus species isolated from marsh and grassland decolourized anthraquinone and azo dyes

(*Submitted to Bioscience, Biotechnology, and Biochemistry*)

Abstract

The biotechnological relevance of the biomolecules produced by Bacillus species remains topical and, may not diminish in the foreseeable future. The enzyme battery produced by members of the Bacillus genus including ligninolytic enzymes has shown immense industrial relevance. Consequently, some bacterial isolates from the marsh and grassland in Hogsback forest reserve of the Eastern Cape Province of South Africa were studied for ligninolytic activities. The utilization and degradation potential of lignin model compounds (guaiacol and veratryl alcohol), and the decolourization of lignin-mimicking dyes: Congo Red (CR) and Remazol Brilliant Blue R (RBBR), were assessed. Isolates with significant ligninolytic potential were identified through 16S rRNA gene sequence analysis, and respective nucleotide sequences were deposited in the GenBank with accession numbers as; *Bacillus* sp. NWODO-3 (KX640919), *Bacillus* sp. MABINYA-1 (KX640920), *Bacillus* sp. MABINYA-2 (KX640921) and *Bacillus* sp. FALADE-1 (KX640922). About 6.53 U mL^{-1} peroxidase activity was recorded against *Bacillus* sp. FALADE-1 while *Bacillus* sp. MABINYA-2 and *Bacillus* sp. NWODO-3 showed 4.08 U mL^{-1} and 3.03 U mL^{-1} peroxidase activity, respectively. *Bacillus* sp. MABINYA-1 produced 1.52 U mL^{-1}. Conversely, maximum dye decolourization was observed with the crude peroxidase from *Bacillus* sp. NWODO-3; CR (69.89 ± 2.64 %) and RBBR (72.12 ± 0.38 %). Dye decolourisation reading for the crude peroxidases from other test isolates are; CR: 55.06 ± 5.48 %, RBBR: 70.45 ± 0.0 % (*Bacillus* sp. MABINYA-1), 42.62 ± 5.55 % and 42.42 ± 4.82 % against CR for *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1, respectively. RBBR was less susceptible to the attack by the crude peroxidase produced by *Bacillus* sp. FALADE-1 and *Bacillus* sp. MABINYA-2 as the dye decolourization activities observed were 1.19 ± 0.0 % and 4.91 ± 0.36 %, respectively. The dye decolourization activities shown by the peroxidases produced by the ligninolytic Bacillus strains were remarkably novel as both *ortho* and *para* arene substituent dyes were decolourized hence, an indication of industrial relevance in bioremediation.

Keywords: Dye decolourization, ligninolytic bacteria, microbial enzyme, peroxidase.

4.1. Introduction

Lignin constitutes a major hassle in the conversion of lignocellulosic-carbonoclastic materials to value-added products. Example of such valorization process is the utilization of lignocellulose as feedstock for biofuel production. Hence, efficient delignification of lignocellulosic biomass is imperative. The biological approach to delignification involves the use of microbes or microbial products such as enzymes (Falade *et al.,* 2017a) due to, perhaps, the mild reaction conditions, increased yield and low energy requirements (Sánchez *et al.,* 2011).

The lignin degradation potentials of several fungal species have been severally studied and their effectiveness vastly documented (Hatakka, 1994; Leonowicz *et al.,* 1999; Martinez *et al.,* 2004; Wan and Li, 2012). However, the emergence of some classes of bacteria as ligninolytic microbes is quite nascent and requires adept combination of biotechnological tools to unravel the immense potentials possessed by the microbes. Some classes of bacteria whose emerging role in lignin degradation has been reported include actinomycetes, α-proteobacteria and γ-proteobactria (Bugg *et al.,* 2011). Similarly, members of the Bacillus genus have been reported to possess ligninolytic abilities (Bandounas *et al.,* 2011; Chang *et al.,* 2014), and these members include *Bacillus* sp. LD003 (Bandounas *et al.,* 2011), *Bacillus* sp. CS-1 and *Bacillus* sp. CS-2 (Chang *et al.,* 2014). The lignin-degradation activities shown by these microbes have been partly attributed to the production of oxidative enzymes, predominantly peroxidases (Falade *et al.,* 2017b).

Besides the delignification activity of peroxidases, the unique oxidative properties of the enzymes have seen other applications including dye decolourization and xenobiotic degradation emerge (Falade *et al.,* 2017a). The high redox potentials of microbial peroxidases and the ability to oxidize recalcitrant phenolic compounds underscore the relevance of these microbes to humanity. Synthetic dyes are recalcitrant to degradation, thus, constitute environmental nuisance upon discharge as industrial effluent. Consequently, the effective removal from the environment through partial or complete degradation remains a challenge.

Physico-chemical treatment approach has been applied for the removal of the noxious substances (dye) from the environment. However, these approaches among others; adsorption and flocculation have high limitations and are inefficient (Singh *et al.,* 2015). The other downside of these techniques includes high operational cost and the creation of secondary pollution (Parshetti *et al.,* 2012). The biological approach, which includes the use of microorganisms and sub-molecules such

as enzymes, in the degradation of dye in effluents, has been effective and it is saddled with less limitations (Falade *et al.*, 2017a). Hence, the exploration of microbial diversity, besides the already known species, for dye decolourization potentials becomes imperative. Consequently, the ligninolytic bacteria species isolated from marsh and grassland of Hogsback forest reserve were evaluated for peroxidase production and the decolourization of dyes with varied arene substituents.

4.2. Materials and methods

4.2.1. Sampling site and sample collection

Samples of decaying wood, soil, sediment, moist rock scrapings and water were collected from marsh and grassland in Hogsback forest reserve of the Raymond Mhlaba Municipality, Eastern Cape, South Africa and transported on ice to Applied and Environmental Microbiology Research Group (AEMREG) Laboratory, University of Fort Hare, Alice, South Africa for analysis.

Hogsback lies on the Amathole Mountains of the Eastern Cape Province of South Africa, with geographical coordinates as S32°.598' E26°.938'. The pristine forest reserve has waterfalls and trout fishing is common. The Hogsback forest reserve has been described as the second-largest per unit area in South Africa comprising indigenous forests, with pockets of Afromontane rain forests covering a large area. Additionally, marsh and grassland are other features of Hogsback.

4.2.2. Isolation of ligninolytic bacteria

Lignin degrading bacteria was isolated using standard techniques (Sasikumar *et al.*, 2014) with slight modification as previously described by Falade *et al.* (2017b).

4.2.3. Lignin degradation assay

The isolates were further evaluated for ligninolytic potential using the modified method of Taylor *et al.* (2012) as described by Falade *et al.* (2017b) in a previous study.

4.2.4. Bacterial identification

Isolates with promising ligninolytic activity were characterized using 16S rRNA gene sequence analysis as previously described elsewhere (Falade *et al.*, 2017b). Phylogenetic analysis was conducted by neighbour-joining method using MEGA 7.0.21 (Kumar *et al.*, 2016).

4.2.5. Peroxidase activity screening

Qualitatively peroxidase activity was determined as described by López *et al.* (2006). Briefly, isolates were inoculated in nutrient agar and incubated at 30 ^{o}C for 48 h. Thereafter, 30 µL of equal parts of 0.4 % (v/v) hydrogen peroxide (H_2O_2) and 1% pyrogallol in water was added to the colony. Colonies with yellow-brown colour were recorded as positive.

4.2.6. Peroxidase production and enzyme preparation

Peroxidase was produced in a submerged fermentation system using the method described by Falade *et al.* (2017b). Fermented broth was centrifuged (15000 rpm) for 10 min at 4 ^{o}C using benchtop cold centrifuge (SIGMA 1-14K). The supernatant was subsequently utilized for peroxidase assay.

4.2.7. Peroxidase activity quantitation

Peroxidase activity was quantified through the measurement of the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin in line with standard methods (Chance and Maehly, 1955; Park, 2006) but with slight modification reported elsewhere (Falade *et al.*, 2017b).

4.2.8 Dye decolourization assay

Lignin-mimicking dyes; Remazol Brilliant Blue R (RBBR) and Congo Red (CR) were assessed for decolourization (Bandounas *et al.,* 2011). About 5 µL of an 18 h culture was aseptically inoculated in dye-agar with the following composition; K_2HPO_4 (4.55 g/L), KH_2PO_4 (0.53 g/L), $MgSO_4$ (0.5 g/L), NH_4NO_3 (5 g/L), yeast extract (0.1 g/L), glycerol (40 mM), dye (100 mg/L RBBR; 50 mg/L CR), and agar (15 g/L). The cultures were incubated at 30°C and examined daily for growth and development of decolourization zones. The total incubation time was 168 h.

Furthermore, the rate of decolourization of the dyes was similarly evaluated in line with the modified method of Kalyani *et al.* (2011). The reaction mixture (400 µL) contained dye (100 mg/L), potassium phosphate buffer (0.1 M, pH 6) and culture supernatant (crude enzyme). The reaction was initiated via the addition of 0.5 % hydrogen peroxide (30 % w/w) and subsequently incubated at 25 ± 2 ^{o}C for 30 min. Absorbance was read at 490 nm and 590 nm being the maximum wavelength for CR and RBBR, respectively (Ollikka *et al.,* 1993) using SynergyMx 96-well

microtitre plate reader (BioTeK Instruments). A reaction mixture without the crude enzyme served as the control. Dye decolourization was measured by monitoring the decrease in absorbance of each dye and expressed as percentage decolourization:

$$\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100\% \qquad \text{.....Eqn. 1.}$$

4.2.9. Data analysis

All data including absorbance readings and halo zones were subjected to analysis of variance (ANOVA) using GraphPad Prism 7 at a 5% ($P \leq 0.05$) confidence interval. Where applicable, results were presented as mean values ± standard deviation (STD).

4.3. Results and discussion

4.3.1. Isolation of ligninolytic bacteria

A total of forty-nine (49) ligninolytic bacteria were isolated from marsh and grassland in Hogsback located in Raymond Mhlaba Municipality, Eastern Cape, South Africa (Table 4.1). The isolates were presumed to be ligninolytic due to their ability to utilize alkali lignin as sole carbon source in an enrichment medium. Isolation of bacteria with ligninolytic potential has previously been reported (Raj *et al.*, 2007; Chandra *et al.*, 2008; Bandounas *et al.*, 2011; Taylor *et al.*, 2012; Chang *et al.*, 2014). However, this is the first-time isolation of ligninolytic bacteria from Hogsback forest reserve in South Africa is being reported. Organisms with ligninolytic potential are promising candidates for delignification of feedstock for bioethanol production (Falade *et al.*, 2017a). They are as well significant in the valorization of lignocellulose to other value-added products. Besides the importance of ligninolytic organisms in the valorization of lignocellulosic biomass, they also have the potential for the production of ligninolytic enzymes including peroxidases with significant prospective industrial applications.

Table 4.1. Colonial morphology of isolated bacteria on alkali lignin plate

S/N	Isolate ID	Isolation source	Form	Pigmentation	Elevation	Margin
1.	HBB1A	Decaying wood	Circular	Creamy	Raised	Entire
2.	HBB1B	Decaying wood	Circular	Yellowish	Raised	Entire
3.	HBB2A	Decaying wood	Circular	Transparent	Flat	Undulate
4.	HBB2B	Decaying wood	Circular	Milky	Raised	Entire
5.	HBB4A	Soil	Circular	Creamy	Flat	Entire
6.	HBB4B	Soil	Irregular	Creamy	Flat	Undulate
7.	HBB4C	Soil	Irregular	Transparent	Flat	Undulate
8.	HBB5A	Soil	Irregular	Creamy	Flat	Undulate
9.	HBB5B	Soil	Irregular	Creamy	Flat	Undulate
10.	HBB6A	Sediment	Circular	Transparent	Raised	Entire
11.	HBB7A	Sediment	Irregular	Creamy	Flat	Undulate
12.	HBB8A	Sediment	Irregular	Brownish	Flat	Undulate
13.	HBB8B	Sediment	Circular	Transparent	Raised	Entire
14.	HBB9A	Soil scrapping	Circular	Creamy	Raised	Entire
15.	HBB10A	Soil scrapping	Circular	Creamy	Raised	Entire
16.	HBB11A	Soil scrapping	Circular	Creamy	Raised	Entire
17.	HBB11B	Soil scrapping	Circular	Creamy	Flat	Entire
18.	HBB12A	Decaying wood	Circular	Creamy	Raised	Entire
19.	HBB13A	Soil scrapping	Circular	Creamy	Raised	Entire
20.	HBB13B	Soil scrapping	Circular	Yellowish	Raised	Entire
21.	HBB13C	Soil scrapping	Circular	Whitish	Raised	Entire
22.	HBB14A	Decaying wood	Irregular	Creamy	Flat	Undulate
23.	HBB15A	Moist soil	Irregular	Whitish	Flat	Undulate
24.	HBB16A	Decaying wood	Irregular	Milky	Flat	Undulate
25.	HBB18A	Decayed wood	Circular	Yellowish	Raised	Entire
26.	HBB19A	Water	Circular	Creamy	Raised	Entire
27.	HBB20A	Water	Circular	Creamy	Raised	Entire
28.	HBB21A	Sediment	Irregular	Whitish	Flat	Undulate
29.	HBB21B	Sediment	Circular	Yellowish	Raised	Entire
30.	HBB22A	Water	Circular	Yellowish	Raised	Entire
31.	HBB22B	Water	Circular	Creamy	Raised	Entire
32.	HBB23A	Sediment	Circular	Yellowish	Raised	Entire
33.	HBB23B	Sediment	Circular	Creamy	Raised	Entire
34.	HBB24A	Sediment	Circular	Yellowish	Raised	Entire

35.	HBB25A	Rock scrapping	Circular	Creamy	Raised	Entire
36.	HBB26A	Waterfall sediment	Circular	Yellowish	Flat	Entire
37.	HBB27A	Waterfall sediment	Circular	Yellowish	Raised	Entire
38.	HBB27B	Waterfall sediment	Irregular	Creamy	Flat	Undulate
39.	HBB28A	Rock scrapping	Circular	Yellowish	Raised	Entire
40.	HBB29A	Rock scrapping	Irregular	Creamy	Flat	Undulate
41.	HBB29B	Rock scrapping	Circular	Yellowish	Raised	Entire
42.	HBB29C	Rock scrapping	Circular	Creamy	Raised	Entire
43.	HBB30A	Decayed wood	Circular	Yellowish	Raised	Entire
44.	HBB31A	Soil particles	Irregular	Transparent	Flat	Undulate
45.	HBB32A	Sediment	Circular	Creamy	Flat	Entire
46.	HBB32B	Sediment	Irregular	Creamy	Flat	Undulate
47.	HBB34A	Decayed wood	Circular	Creamy	Raised	Entire
48.	HBB35A	Moist decayed wood	Irregular	Transparent	Flat	Undulate
49.	HBB35B	Moist decayed wood	Irregular	Creamy	Flat	Undulate

4.3.2. Lignin degradation potential

The ligninolytic potential of the isolates was also assessed using their ability to utilize and degrade some lignin model compounds including 2-methoxyphenol (guaiacol) and 3,4-Dimethoxybenzyl alcohol (veratryl alcohol). Lignin model compounds and aromatic monomers such as guaiacol and veratryl alcohol are usually used for screening of organisms for ligninolytic potentials (Bandounas *et al.,* 2011; Taylor *et al.,* 2012). Table 4.2 showed that 13 isolates (26.5 %) were able to utilize and degrade both guaiacol and veratryl alcohol. The degree of degradation of the compounds by the isolates was measured by the diameter of the halo zone with isolate HBB1A having the highest zone of degradation for the two compounds (guaiacol: 42.0 ± 0.0 mm, veratryl alcohol: 41.0 ± 1.0 mm) and HBB29C having the least zone of degradation (guaiacol: 9.0 ± 1.0 mm, veratryl alcohol: 7.0 ± 1.0 mm). The degraded part was revealed as a clear zone around the bacterial colony on plate while the brown colouration of the un-degraded part resulted from the interaction of hydrogen iodide (generated from dissolving potassium iodide and iodine in water) with the two aromatic alcohols in the presence of oxygen after being flooded with Gram's iodine (Falade *et al.,* 2017b) at 168 h of incubation. The clear zone around the bacterial colony in this study could either be as a result of utilization of the compounds or their degradation by extracellular enzymes including lignin modifying enzymes. Furthermore, the capability of bacterial strains to utilize and degrade

guaiacol and veratryl alcohol as observed in this study might also be attributed in part to the activity of alcohol dehydrogenase which is required to oxidize aromatic alcohols.

The ligninolytic activity of certain aromatic compound degraders including *Pseudomonas* sp. and *Burkholderia* sp. has been reported (Bandounas *et al.*, 2011). However, our result contradicts the findings of Bandounas *et al.* (2011) who reported that certain bacterial strains including *Pandoraea norimbergensis* LD001, *Pseudomonas* sp. LD002 and *Bacillus* sp. LD003 were not able to utilize or degrade the alcoholic forms of the aromatic monomers investigated including guaiacol and veratryl alcohol. The seemingly discrepancies may be due to ecological reasons as strains studied by Bandounas *et al.* (2011) were isolated from soil beneath decomposing wood logs in the Netherlands while the bacterial isolates used in this study were from different environments in South Africa. In order to clearly understand these discrepancies, the metagenomics analysis of the samples is imperative.

Table 4.2. Lignin degradation potential of bacterial isolates

S/N	Positive isolates	Diameter of halo zone for GA (mm)	Diameter of halo zone for VA (mm)
1.	HBB1A	42.0 ± 0.0^{a}	41.0 ± 1.0^{a}
2.	HBB1B	40.0 ± 2.0^{a}	39.0 ± 1.0^{a}
3.	HBB4A	24.0 ± 0.0^{b}	23.0 ± 1.0^{b}
4.	HBB5A	26.0 ± 0.0^{c}	29.0 ± 1.0^{c}
5.	HBB5B	20.0 ± 0.0^{d}	25.0 ± 1.0^{d}
6.	HBB7A	22.0 ± 2.0^{d}	32.0 ± 0.0^{c}
7.	HBB10A	12.0 ± 2.0^{e}	13.0 ± 1.0^{e}
8.	HBB11B	18.0 ± 2.0^{f}	20.0 ± 0.0^{f}
9.	HBB22A	31.0 ± 1.0^{g}	21.0 ± 1.0^{f}
10.	HBB29A	31.0 ± 1.0^{g}	34.0 ± 0.0^{g}
11.	HBB29B	30.0 ± 2.0^{g}	34.0 ± 0.0^{g}
12.	HBB29C	9.0 ± 1.0^{h}	7.0 ± 1.0^{h}
13.	HBB30A	17.0 ± 1.0^{i}	10.0 ± 0.0^{i}

GA: Guaiacol; VA: Veratryl Alcohol. Values represent mean ± standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different ($P > 0.05$).

4.3.3. Bacterial identity

Four bacterial isolates with promising ligninolytic potential (HBB5A, HBB5B, HBB7A and HBB29A) were identified using 16S rRNA gene sequence analysis and the results showed that all the isolates belong to Bacillus genus. The results of the BLAST search of the respective nucleotide sequences in the NCBI database revealed that HBB5A, HBB5B and HBB29A had 99 % similarity to *Bacillus lentus* strain FJAT-10603 (JN450800), *Bacillus* sp. strain NC62 (KY454505) and *Bacillus* sp. strain FJAT-25753 (KR077842) respectively. However, HBB7A was 100% similar to *Bacillus* sp. strain HP5F2 (KM187486) and [Brevibacterium] frigoritolerans strain Hb-1 (KC139406) as it also showed 99 % similarity to several strains of *Bacillus* species. The respective 16S rRNA gene sequences of the identified organisms are available in the NCBI database with the following names and accession numbers: HBB5A: *Bacillus* sp. strain NWODO-3 (KX640919), HBB5B: *Bacillus* sp. strain MABINYA-1 (KX640920), HBB7A: *Bacillus* sp. strain MABINYA-2 (KX640921) and HBB29A: *Bacillus* sp. strain FALADE-1 (KX640922). The unrooted phylogenetic tree indicating the evolutionary relationships of the Bacillus strains in this study with other Bacillus species available in the NCBI database is shown in Fig. 4.1. The evolutionary analysis shows that Bacillus strains studied (indicated with black tips) are more closely related to one another than other Bacillus species.

The identity of these organisms as *Bacillus* spp. further confirms the emerging role of Bacillus species in lignin degradation. This finding is consistent with previous studies on the ligninolytic activities of Bacillus species where Bandounas *et al.* (2011) reported the ligninolytic potential of *Bacillus* sp. LD003 isolated from soil beneath decomposing wood logs in Netherlands. Recently, Chang *et al.* (2014) also reported the lignin-degrading activity of *Bacillus* sp. CS-1 and *Bacillus* sp. CS-2 from forest soils in Japan. However, this is perhaps the first report of ligninolytic Bacillus species from Hogsback forest in South Africa. The emerging ligninolytic activity of Bacillus species confers on this class of bacteria the potential for application in biological delignification of lignocellulosic biomass, consequently enhancing valorization of lignocellulosic biomass to value added products of economic importance.

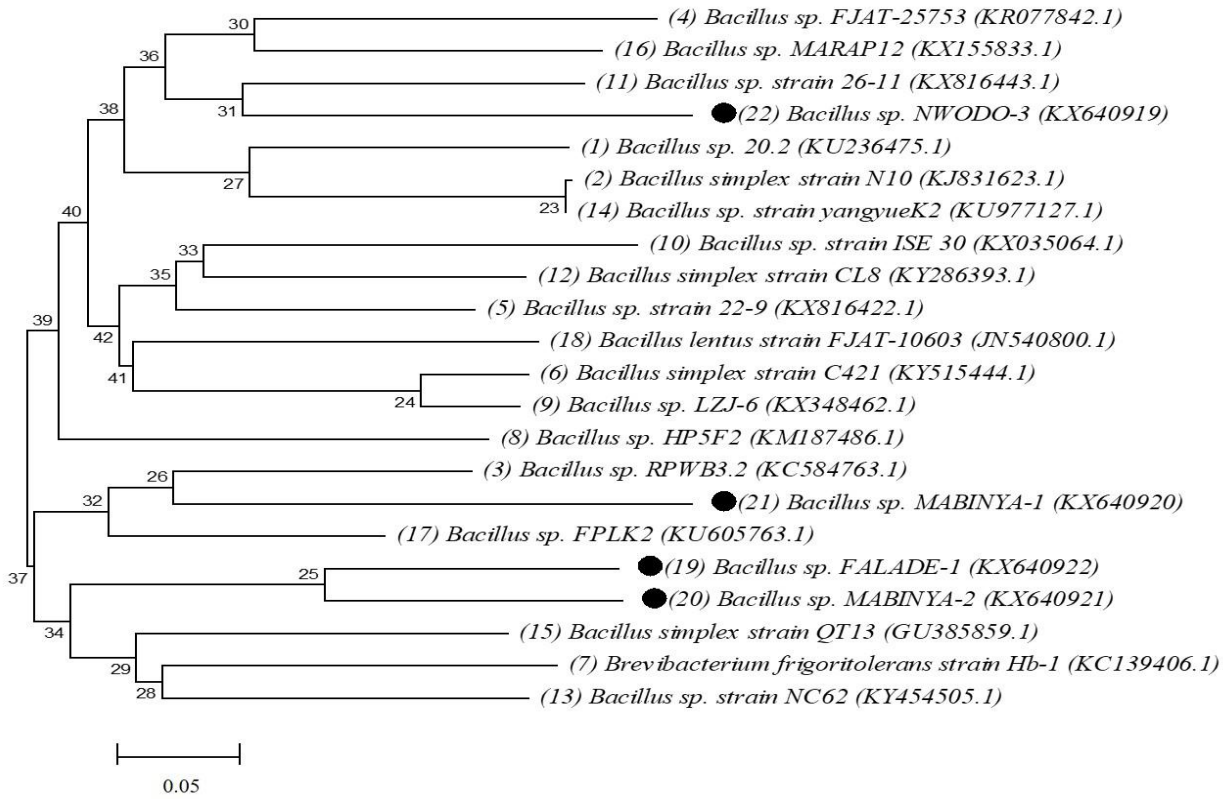

Fig. 4.1. Phylogenetic tree showing the evolutionary relationships of ligninolytic Bacillus strains in this study and selected Bacillus species in the NCBI database. The ligninolytic bacteria isolated and sequenced in this study are shown in black tips with ID Numbers 19-22. The bootstrap values of 1000 replicates are shown next to the branches while accession numbers are indicated in parentheses.

4.3.4. Peroxidase production by novel ligninolytic Bacillus species

Bacillus species have been described as one of the most significant industrial producers of enzymes partly owing to their capacity to produce large amount of extracellular enzymes (Schallmey *et al.*, 2004). To assess the potential of the newly isolated ligninolytic Bacillus species to produce extracellular peroxidases, the qualitative peroxidase activity of the organisms was determined. The results revealed that all the ligninolytic Bacillus species assessed showed peroxidase activity on plate (Fig. 4.2) with the presence of yellowish-brown colouration on the bacterial colony after its reaction with 0.4 % (v/v) hydrogen peroxide (H_2O_2) and 1 % pyrogallol at 48 h of incubation on nutrient agar (Lopez *et al.*, 2006). To further evaluate the peroxidase production potentials of these

organisms, the level of extracellular peroxidase produced by the ligninolytic Bacillus species was determined using a quantitative assay as described in the method and the result is presented in Table 4.3. *Bacillus* sp. FALADE-1 exhibited the highest potential for peroxidase production with 6.53 U mL^{-1} followed by *Bacillus* sp. MABINYA-2 and *Bacillus* sp. NWODO-3 with 4.08 U mL^{-1} and 3.03 U mL^{-1} of peroxidase respectively while *Bacillus* sp. MABINYA-1 produced 1.52 U mL^{-1} of peroxidase. However, all the ligninolytic Bacillus species in this study showed promising potential for peroxidase production.

The quantity of peroxidase produced by these organisms is higher than what was reported by Rao and Kavya (2014) and Musengi *et al.* (2014) where *Bacillus subtilis* and *Streptomyces* sp. BSII#1 produced 0.00045 U mL^{-1} and 1.30 U mL^{-1} of peroxidase, respectively. This indicates that the studied Bacillus strains hold a high potential for enhanced peroxidase production.

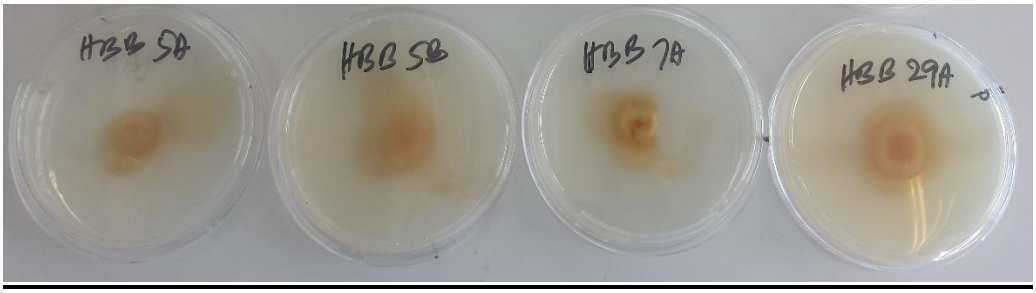

Fig. 4.2. Qualitative peroxidase activity screening. HBB5A: *Bacillus* sp. NWODO-3, HBB5B: *Bacillus* sp. MABINYA-1, HBB7A: *Bacillus* sp. MABINYA-2, HBB29A: *Bacillus* sp. FALADE-1.

Table 4.3. Peroxidase production by novel ligninolytic Bacillus species

S/N	**Organisms**	**Peroxidase activity (U mL^{-1})**
1.	*Bacillus* sp. NWODO-3	3.03
2.	*Bacillus* sp. MABINYA-1	1.52
3.	*Bacillus* sp. MABINYA-2	4.08
4.	*Bacillus* sp. FALADE-1	6.53

4.3.5. Dye decolourization

Decolourization of lignin-like dyes such as Azure B, Methylene Blue, Toluidine Blue O, Malachite Green, Indigo Carmine, Remazol Brilliant Blue R, Congo Red etc. has been used to determine the ligninolytic potential of bacteria (Bandounas *et al.*, 2011). To further confirm the ligninolytic potential of the isolates, the thirteen (13) positive isolates for the degradation of lignin model compounds (guaiacol and veratryl alcohol) were evaluated for their abilities to decolourize two ligninolytic indicator dyes: Remazol Brilliant Blue R (RBBR) and Congo Red (CR), with different arene substituent attachment positions. RBBR is a recalcitrant anthraquinone dye with its aromatic substituent attached at the *para* position of its structural backbone (anthraquinone) while CR is an azo dye having its two azo groups (-N=N-) which serve as the chromophore attached at the *ortho* position. The position of attachment of the various arene rings might probably contribute to the recalcitrance of synthetic dyes to degradation. Consequently, degradation of dyes with *ortho, meta* and *para* arene substituents would suffice as a novel mechanism of dye degradation.

The results of the qualitative assessment of the bacterial isolates for dye decolourization as revealed in Table 4.4 showed that 5 isolates (38.46 %) decolourized RBBR while 11 isolates (84.62 %) were positive for decolourization of CR within 72 h. This finding suggests that CR (azo dye) is more susceptible to decolourization than RBBR, an athraquinone dye. However, only *Bacillus* sp. NWODO-3, *Bacillus* sp. MABINYA-1, *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1 were able to decolourize both RBBR and CR in this study. The dye decolourization observed in this study could either be as a result of dye adsorption to the bacterial colonies or oxidation of the dyes by enzymes (laccases and peroxidases) secreted by the bacteria.

Table 4.4. Qualitative assessment of bacteria for dye decolourization

S/N	Isolates	RBBR	CR
1.	HBB1A	-	+
2.	HBB1B	-	+
3.	HBB4A	-	+
4.	HBB5A	+	+
5.	HBB5B	+	+
6.	HBB7A	+	+
7.	HBB10A	+	-
8.	HBB11B	-	+
9.	HBB22A	-	+
10.	HBB29A	+	+
11.	HBB29B	-	-
12.	HBB29C	-	+
13.	HBB30A	-	+

+: positive; -: negative; RBBR: Remazol Brilliant Blue R; CR: Congo Red.

Furthermore, enzyme-based dye decolourization is of greater interest, partly, due to its efficiency, greater specificity and non-dependence on the growth rates of organisms (Husain *et al.,* 2009; Kalyani *et al.,* 2011). The involvement of microbial enzymes including peroxidases, laccases and azo reductase in biodegradation of dyes has been suggested (Kalyani *et al.,* 2011). Moreover, crude and purified forms of bacterial peroxidase have been used in dye decolourization (Dawkar *et al.,* 2009; Ghodake *et al.*, 2009). Therefore, the potential activity of crude peroxidase produced by the new ligninolytic Bacillus species in dye decolourization was evaluated by incubating the culture supernatant from the organisms with the dyes (Congo Red-CR and Remazol Brilliant Blue R-RBBR) at 25 ± 2 ^{o}C for 30 min in a reaction mixture containing phosphate buffer (pH 6) and hydrogen peroxide. The results (Fig. 4.3) revealed that crude peroxidase from *Bacillus* sp. NWODO-3 showed the highest decolourization activity for both CR (69.89 ± 2.64 %) and RBBR (72.12 ± 0.38 %) followed by peroxidase from *Bacillus* sp. MABINYA-1 (CR: 55.06 ± 5.48 %, RBBR: 70.45 ± 0.0 %). However, there was no significant difference ($P>0.05$) in the

decolourization of CR by peroxidases from *Bacillus* sp. MABINYA-2 (42.62 ± 5.55 %) and *Bacillus* sp. FALADE-1 (42.42 ± 4.82 %). Although there was a significant difference ($P<0.05$) in the decolourization of RBBR by peroxidases from *Bacillus* sp. MABINYA-2 (4.91 ± 0.36 %) and *Bacillus* sp. FALADE-1 (1.19 ± 0.0 %), their activities were insignificant. The disparity observed in the decolourization of CR and RBBR by peroxidases from the *Bacillus* species may probably be due to the structural variation of the dyes. The result indicates that crude peroxidases from *Bacillus* sp. NWODO-3 and *Bacillus* sp. MABINYA-1 showed the best potential activity for arene substituent dye decolourization. This finding is comparable to previous related study by Kalyani *et al.* (2011) who reported the decolourization of various textile dyes (such as methyl orange, reactive red 2, reactive blue 59 etc.) by peroxidase from *Pseudomonas* sp. SUK 1 with highest decolourization activity of 72% on methyl orange.

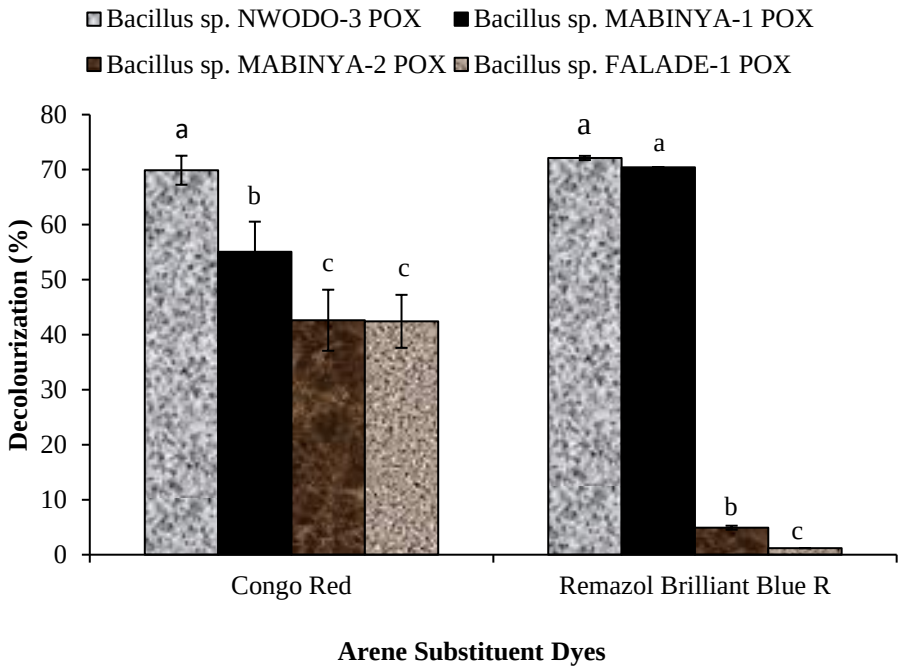

Fig. 4.3. Decolourization of anthraquinone and azo dyes by peroxidases from new Bacillus strains. Error bars with the same alphabet are not significantly different ($P> 0.05$). POX: Peroxidase.

4.4. Conclusion

In conclusion, the four novel ligninolytic Bacillus strains identified in this study have shown promising potentials for delignification and production of peroxidases with industrial relevance in bioremediation. The ability of peroxidases from *Bacillus* sp. NWODO-3 and *Bacillus* sp. MABINYA-1 to decolourize dyes with *ortho* and *para* arene substituents confers on them the potential for application in textile effluent treatment and synthetic dye transformation. However, detailed characterization of the enzymes is imperative for practical applications.

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CHAPTER FIVE

Optimized peroxidase production and detection of catalase-peroxidase gene (*KatG*) in a Bacillus species isolated from Hogsback forest reserve, South Africa

(*Submitted to Enzyme and Microbial Technology*)

Abstract

This study sought to optimize peroxidase production by a bacillus species (*Bacillus* sp. FALADE-1-KX640922) isolated from Hogsback forest reserve in South Africa and determine the gene encoding peroxidase activity in the organism. We optimized peroxidase production by manipulating the environmental and nutritional parameters under submerged fermentation. Subsequently, the gene encoding peroxidase activity was determined using nested polymerase chain reaction and Sanger DNA sequencing. Optimum peroxidase production by *Bacillus* sp. FALADE-1 was attained at pH 8, 30 ^{o}C and 150 rpm. The addition of guaiacol to lignin fermentation medium enhanced peroxidase production by over 100 % in *Bacillus* sp. FALADE-1. However, the other lignin model compounds (veratryl alcohol, vanillin, vanillic acid and feriluc acid) repressed the enzyme production by the organism. Supplementation of the fermentation medium with ammonium sulphate gave the maximum peroxidase yield (8.87 U mL^{-1}). Under optimized conditions, the maximum peroxidase production by *Bacillus* sp. FALADE-1 was attained at 48 h (8.32 U mg^{-1}). Interestingly, the blast search of the nucleotide sequence of the amplified gene in PeroxiBase database showed 100% similarity to *Sporotrichum thermophile* catalase-peroxidase gene (*katG*), as well, the deduced protein sequence clustered with bacterial catalase-peroxidases and had a molecular weight of 11.445 kDa with 7.01 as the isoelectric point. Subsequently, the nucleotide sequence was deposited in the GenBank as *Bacillus* sp. FALADE-1 catalase-peroxidase gene under the accession number MF407314. In conclusion, peroxidase production by *Bacillus* sp. FALADE-1 increased by 2.22-fold as *katG* seemed to be responsible for the peroxidase activity expressed in the organism.

Keywords: Bifunctional enzyme, catalase-peroxidase, enzyme production, ligninolytic enzymes, optimization, peroxidase.

5.1. Introduction

Oxidative enzymes are topical perhaps, due to, their biotechnological potentials in various industrial biocatalytic processes including but not limited to bioremediation, biodegradation, delignification, biobleaching and biopulping.

Peroxidases, are major oxidative enzymes which have recently received increased attention, probably, owing to their high redox potentials and the ability to oxidize recalcitrant compounds (Falade *et al.,* 2017a). These characteristics have motivated for their application potentials in the development of biosensors and diagnostic kits for detection of hydrogen peroxide (Jia *et al.,* 2002; Agostini *et al.,* 2002), decolourization of synthetic dyes (Kalyani *et al.,* 2011), development of skin-lightening agents (Draelos, 2015; Falade *et al.,* 2017a) and removal of emerging pollutants and micropollutants in wastewater (Taboada-Puig *et al.,* 2015).

Nevertheless, the industrial applicability of peroxidases is being hampered by inadequate amount of enzyme produced and high cost of production (Ferrer *et al.,* 1991; Torres *et al.,* 2003). More so, the commercially available peroxidases such as horseradish peroxidase (HRP), peroxidase from *Bjerkandera adusta* and streptavidin: peroxidase from *Streptomyces avidinii* could not probably meet the increasing industrial demand for peroxidases. Hence, the imperativeness of new sources of peroxidase to satisfy the growing industrial demands.

Production of peroxidases from plant and white-rot fungi have been extensively studied (Lavery *et al.,* 2010; Kharatmol and Pandit, 2012; Rathnamsamy *et al.,* 2014; Kong *et al.,* 2016; Zhang *et al.,* 2016). Peroxidase production by a number of bacteria, predominantly, actinobacteria (Tuncer *et al.,* 2009; Nour El-Dein *et al.,* 2014; Musengi *et al.,* 2014) and very few belonging to Bacillus genus (Dawkar *et al.,* 2009; Rajkumar *et al.,* 2013; Patil, 2014; Rao and Kavya, 2014) have also been reported. Moreover, bacteria seem to be more promising for enhanced peroxidase production, perhaps, due to their high genetic maneuverability which is difficulty in fungi. More so, detection of the gene encoding peroxidase in bacteria is another step that could be explored for improved peroxidase production through molecular optimization and genetic engineering.

Bacillus species are described as the “major workhorse industrial microorganisms” (Schallmey *et al.,* 2004), with enhanced enzyme production potential. They are characterized by high growth rate, ability to produce extracellular proteins in large quantity and general safety (Schallmey *et al.,* 2004). Moreover, Bacillus species have shown great potential for production of various

extracellular enzymes (Sevinc and Demirkan, 2011; Barros *et al.*, 2013; Pant *et al.*, 2015). Also, some Bacillus species have been employed for production of cellulolytic and pectinolytic enzymes (Soares *et al.*, 2001; Dias *et al.*, 2014; Padilha *et al.*, 2015). Nonetheless, there is dearth of information on the production of ligninolytic enzymes, particularly, peroxidases by Bacillus species. Therefore, this study seeks to optimize peroxidase production by *Bacillus* sp. FALADE-1 (KX640922) and determine the gene encoding peroxidase activity in the organism.

5.2. Materials and methods

5.2.1. Organism source, screening and identification

The organism (*Bacillus* sp. FALADE-1) was isolated from rock scrappings collected from Hogsback forest reserve in Eastern Cape, South Africa using the method described by Sasikumar *et al.* (2014) with some modifications. Then, the organism was screened for ligninolytic and peroxidase production potentials [Unpublished results] and subsequently identified as a *Bacillus* sp. using 16S rDNA partial sequence analysis.

5.2.2. Enzyme production using submerged fermentation technique

Submerged fermentation for peroxidase production was carried out as described by Falade *et al.* (2017b) where 100 mL of the fermentation medium: K_2HPO_4 (4.55 g L^{-1}), KH_2PO_4 (0.53 g L^{-1}), $MgSO_4$ (0.5 g L^{-1}), NH_4NO_3 (5 g L^{-1}), yeast extract (0.1 g L^{-1}) and 0.1 % w/v lignin (Sigma-Aldrich, SA) was aseptically inoculated with 2 % inoculum of the bacterial suspension in normal saline (O.D. 600 nm $\approx$ 1.0) at pH 7 using uninoculated media as control. The culture was subsequently incubated at 30 ^{o}C and 140 rpm for 48 h, afterwards, the crude enzyme was prepared as reported by Falade *et al.* (2017b).

5.2.3. Enzyme activity assay

Peroxidase production was evaluated by determining the peroxidase activity using the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin as described by Park (2006) with some modifications reported by Falade *et al.* (2017b).

5.2.4. Optimization studies on peroxidase production by Bacillus sp. FALADE-1

The optimized conditions for peroxidase production by the Bacillus species were determined as described by Fatokun *et al.* (2016). Briefly, peroxidase production was optimized by adjusting the various culture conditions such as pH, temperature and agitation rate as well as manipulating the fermentation medium composition using conventional method. The observed optimum parameters were then employed for subsequent fermentation (Fatokun *et al.,* 2016).

5.2.4.1. Determination of initial pH for optimum peroxidase production

The initial medium pH for optimum peroxidase production was determined by growing the Bacillus strain in a lignin fermentation medium with varying pH (3 to 11), which was adjusted using 1M HCl or 1M NaOH. The culture was subsequently incubated at the conditions earlier described.

5.2.4.2. Determination of incubation temperature for optimum peroxidase production

The incubation temperature for optimum peroxidase production was determined by cultivating the test strain in a lignin fermentation medium with initial pH 8; and incubating the culture at temperatures ranging from 20 to 45 ^{o}C at 5 ^{o}C intervals while other conditions earlier described remained constant.

5.2.4.3. Determination of agitation rate for optimum peroxidase production

The agitation rate for optimum peroxidase production by the test bacteria was determined by incubating the culture at static and different agitation rates ranging from 50 – 200 rpm at 50 rpm interval using the predetermined-optimized culture conditions (pH: 8, temperature: 30 ^{o}C) for 48 h.

5.2.4.4. Effect of lignin model compounds on peroxidase production

The bacteria strain under investigation was cultivated in a lignin fermentation medium supplemented with 1 mmol L^{-1} of different lignin model compounds (guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid) with the potential for induction of lignin modifying enzymes (Musengi *et al.,* 2014) using the predetermined-optimized culture conditions (pH 8, 30 ^{o}C, 150 rpm) for 48 h. The lignin fermentation medium without the supplements served as the control.

5.2.4.5. Effect of nitrogen supplementations on peroxidase production

The effect of nitrogen supplementations on peroxidase production by the Bacillus strain was determined by cultivating the test bacteria in a lignin fermentation medium where yeast extract was supplemented with different inorganic nitrogen sources (ammonium nitrate, ammonium chloride and ammonium sulphate) at the pre-optimized fermentation conditions for 48 h. The medium without nitrogen supplementation was used as the control.

5.2.5. Kinetics of enzyme production and bacterial growth

The kinetics of peroxidase production by the Bacillus strain and the cell growth were determined using the modified method described by Tuncer *et al.* (1999). Briefly, the strain was grown in a lignin fermentation medium under optimized culture conditions for 120 h. Thereafter, the culture was withdrawn every 24 h (Tuncer *et al.,* 1999) and assayed for peroxidase activity. As well, the cell growth was monitored by measuring the optical density (OD) of the culture at 600 nm.

5.2.6. Estimation of extracellular protein

The extracellular protein produced by the test organism was estimated using Bradford method (Bradford, 1976). Briefly, 250 µL of Bradford reagent was added to 10 µL of the supernatant in a 96-well microtitre plate while 10 µL of distilled water was used to replace the sample in the blank. Then, the mixture was incubated at room temperature (25 ± 2 ^{o}C) for 15 min. Subsequently, the absorbance was measured at 595 nm using SynergyMx 96-well microtitre plate reader (BioTeK Instruments, USA). The concentration of the secreted protein was later extrapolated from the curve constructed using bovine serum albumin (BSA) as standard protein. The specific enzyme activity was expressed as U mg^{-1} protein.

5.2.7. DNA extraction

Genomic DNA was extracted from the test organism using boiling method as described by Maugeri *et al.* (2006). A number of colonies of the test strain was suspended in 200 µL of nuclease-free water and heated at 100 ^{o}C for 10 min using Dri Block DB-BD (TECHNE, Lasec, SA). Thereafter, the mixture was centrifuged at 20000 x g for 5 min (HERMLE Z 233 M-2, Lasec, SA) and the recovered supernatant was used as DNA template for polymerase chain reaction (PCR).

5.2.8. Nested PCR

The target gene was amplified using the set of primers listed in Table 5.1 in a nested PCR assay. The oligonucleotide primers which were designed using the DNA sequence of *Bacillus* sp. ABP14 (CP017016), were synthesized by Inqaba Biotech, South Africa. A total of 25 µL reaction mixture which comprised 12.5 µL of master mix (BioLabs, SA), 1µL each of both forward (Baprx F1) and reverse (Baprx R) primers, 5.5 µL of nuclease-free water and 5 µL of extracted DNA was used for the first PCR assay under the following optimized conditions: initial denaturation at 95 ^{o}C (5 min), denaturation at 94 ^{o}C (1 min), annealing at 55 ^{o}C (1 min), extension at 72 ^{o}C (1 min) for 35 cycles and final extension at 72 ^{o}C (5 min). The amplicon from the first PCR assay was then used as the DNA template for the second PCR experiment using the second forward primer, Baprx F2 and the reverse primer, Baprx R under the same PCR conditions in a thermocycler apparatus (G-STORM, UK). Thereafter, the amplified product was visualized by electrophoresis using a 1.5 % agarose gel (Merck, SA) stained with ethidium bromide (Sigma-Aldrich, SA) at 100 V for 45 min in 0.5X TBE buffer through ultraviolet illumination (Alliance 4.7, France). The PCR product was subsequently analysed after being purified, using Sanger dideoxy sequencing method followed by a BLAST search in PeroxiBase database (peroxibase.toulouse.inra.fr) using blastn.

Table 5.1. List of oligonucleotide primers for detection of heme-peroxidase gene in *Bacillus* sp.

Primer name	**Primer sequence (5'-3')**	**Target gene**	**Expected band size (bp)**	**Reference**
Baprx F1	GCAAAAAAGGGCAGTCACGCAA	*Hprx*	465/461	This study
Baprx F2	AAAAGGGCAGTCACGCAATGTA			
Baprx R	TTGAAGAACATCGTCAGCGAATAAT			

5.2.9. Phylogenetic analysis

Phylogenetic analysis of the deduced protein sequence from *Bacillus* sp. FALADE-1 peroxidase gene and selected bacterial heme-peroxidases in the PeroxiBase was conducted using neighbour-joining method (Saito and Nei, 1987) in MEGA 7.0 software (Kumar *et al.,* 2016) while the physicochemical properties were determined using geneious 10.2.2.

5.2.10. Data analysis

Data were analyzed using one-way ANOVA and Tukey's Multiple Comparison Test where applicable. Significance was accepted at $P \leq 0.05$.

5.3. Results and discussion

Given the diverse applications of peroxidase in various industrial processes, enhancement of its production has become imperative. This has therefore necessitated the search for novel sources with increased production capacity. In this study, we assessed peroxidase production by a Bacillus strain isolated from Hogsback forest reserve in the Eastern Cape, South Africa under optimized culture conditions including initial medium pH, incubation temperature, and agitation rate. Also, the composition of the production medium was amended for optimum peroxidase production by the organism. Subsequently, the kinetics of enzyme production and bacterial growth were assessed as the gene encoding peroxidase activity was also determined.

The peroxidase production was determined in this study by measuring the peroxidase activity of the supernatant recovered from the organisms.

5.3.1. Determination of initial pH for optimum peroxidase production

The pH of the cultivation environment exerts a significant influence on microbial growth and metabolism (Saini *et al.*, 2014) as nutrient absorption is determined by the charge on the microbial cells (Salehizadeh and Shojaosadati, 2001). It is therefore important to determine the medium pH that is most favourable for metabolic activities of an organism. The results of the initial medium pH for optimum peroxidase production by *Bacillus* sp. FALADE-1 as presented in Fig. 5.1 showed that the organism was able to produce peroxidase over a wide pH range (5.0 – 10.0) with optimum peroxidase production observed at pH 8 (3.15 U mL^{-1}). However, no peroxidase activity was detected at pH 3, 4 and 11. Although there exists a significant difference ($P < 0.05$) in peroxidase production by *Bacillus* sp. FALADE-1 at the optimal pH when compared to other pH values, there was no significant difference ($P > 0.05$) in peroxidase production by the organism at pH 6, 7, 9, 10 respectively. This finding agrees with previous related study by Rajkumar *et al.* (2013), in which optimum peroxidase production by a *Bacillus* sp. was recorded at pH 8. However, Rao and Kavya (2014) reported pH 6 as the optimal for peroxidase production by *Bacillus subtilis*. This

indicates that pH plays a significant role in peroxidase production (McCarthy, 1987; Niladevi and Prema, 2008).

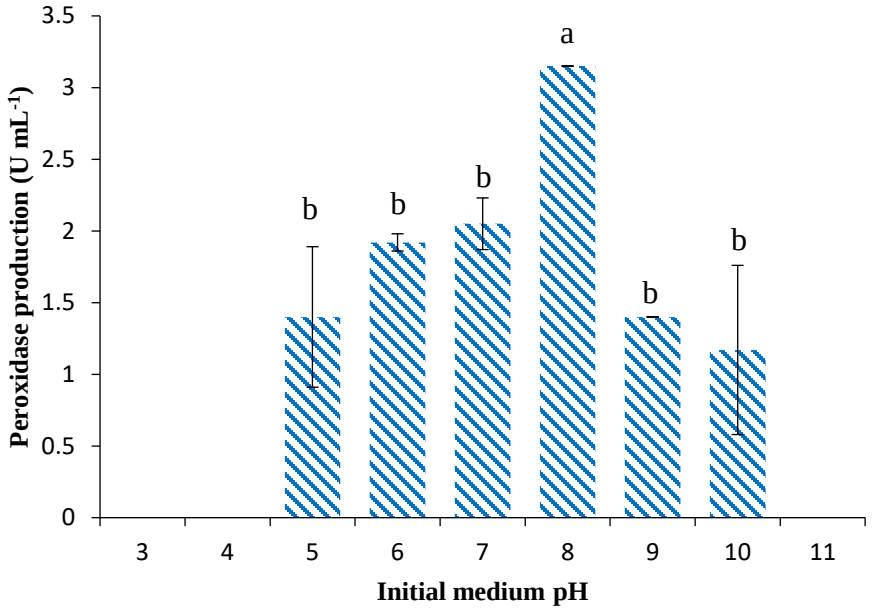

Fig. 5.1. Determination of initial pH for optimum peroxidase production by *Bacillus* sp. FALADE-1. Each column represent mean ± standard deviation. Error bars with the same alphabet are not significantly different (*P> 0.05*).

5.3.2. Determination of incubation temperature for optimum peroxidase production

Microorganisms can grow only within certain limits of temperatures. This environmental factor tends to influence the growth rate, macromolecular composition, levels of intracellular metabolites and enzyme production. It is therefore expedient to determine the temperature that best supports the growth of an organism and optimum enzyme production.

The incubation temperature for optimum peroxidase production by the Bacillus strain is presented in Fig. 5.2. The results showed a significant difference *(P < 0.05)* in peroxidase production across the temperatures: 20 – 45 ^{o}C with optimum peroxidase production observed at 30 ^{o}C (3.15 U mL^{-1}). However, there was no significant difference *(P > 0.05)* in peroxidase production at 20, 35 and

45 ^{o}C. Nevertheless, there was a significant decrease *(P < 0.05)* in peroxidase production at lower and higher temperatures, suggesting a reduction in metabolic activities of the organism (Tandon and Sharma, 2014), which may subsequently inhibit its growth and enzyme biosynthesis (Ray *et al.*, 2007).

This finding is in accordance with the work of Rajkumar *et al.* (2013) where optimum peroxidase production by a *Bacillus* sp. was also attained at 30 ^{o}C. On the contrary, Rao and Kavya (2014) reported optimum peroxidase production by *Bacillus subtilis* at 37 ^{o}C. Moreover, optimum peroxidase production by other bacterial species at 37 and 40 ^{o}C have been reported (Nour El-Dein *et al.*, 2014; Musengi *et al.*, 2014), indicating that incubation temperature for optimum peroxidase production may be strain-dependent (Gautam *et al.*, 2011).

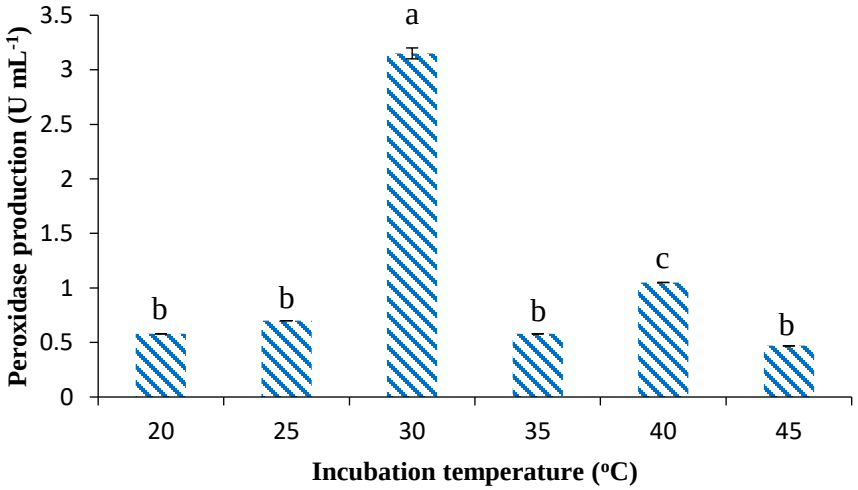

Fig. 5.2. Determination of incubation temperature for optimum peroxidase production by *Bacillus* sp. FALADE-1. Each column represent mean ± standard deviation. Error bars with the same alphabet are not significantly different (*P> 0.05*).

5.3.3. Determination of agitation rate for optimum peroxidase production

Agitation is another critical factor that affects microbial growth and secretion of extracellular enzymes, as it is accountable for aeration and even distribution of nutrients during fermentation

(Satyanarayana and Adhikari, 2006). To improve peroxidase production by the test organism, it is therefore important to determine the agitation rate that is most suitable for optimum peroxidase production by the bacteria. The agitation rate for optimum peroxidase production by *Bacillus* sp. FALADE-1 is presented in Fig. 5.3. The results revealed a significant difference ($P < 0.05$) in peroxidase production by the organism, in static condition, and across all agitation rates (50-200 rpm), with the optimum peroxidase production observed at agitation rate of 150 rpm (3.04 U mL^{-1}). In other words, peroxidase production by the test strain was more favourable at a high agitation rate. In this case, optimum peroxidase production may be attributed to increased aeration of the cultivation medium which could lead to sufficient supply of dissolved oxygen in the medium (Kumar and Takagi, 1999; Sepahy and Jabalameli, 2011) as well as increased nutrient uptake by the organism (Sepahy and Jabalameli, 2011; Beg *et al.*, 2003). The decrease observed in peroxidase production at agitation rate higher than 150 rpm, may probably, be due to enzyme denaturation (Geok *et al.*, 2003) as high agitation may result in cell damage, consequently reducing the number of peroxidase producers.

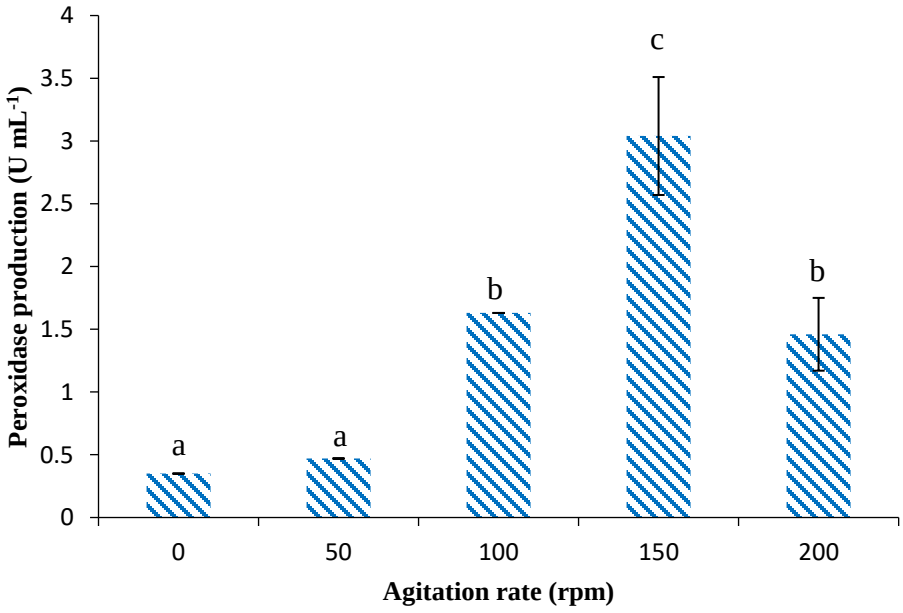

Fig. 5.3. Determination of agitation rate for optimum peroxidase production by *Bacillus* sp. FALADE-1. Each column represent mean ± standard deviation. Error bars with the same alphabet are not significantly different ($P > 0.05$).

This finding contradicts previous related study by Patil (2014) who reported 180 rpm as the optimal agitation rate for lignin peroxidase production by *Bacillus megaterium.* Moreover, several other studies have reported different optimal agitation rates for production of different lignocellulolytic enzymes by different Bacillus strains. Sepahy *et al.* (2011) reported maximum xylanase production by *Bacillus mojavensis* AG137 at 200 rpm. However, Fatokun *et al.* (2017) recorded 50 rpm and 150 rpm as agitation rates for optimum production of xylanase and cellulase by *Bacillus* sp. SAMRC-UFH9, respectively.

5.3.4. Effect of lignin model compounds on peroxidase production

Some lignin model compounds including guaiacol (GA), veratryl alcohol (VALC), vanillin (VAN), vanillic acid (VA) and ferulic acid (FA) are known to induce production of lignin modifying enzymes by microorganisms. In this study, the lignin fermentation medium for peroxidase production was supplemented with 1 mmol L^{-1} of the different lignin model compounds and the results are presented in Fig. 5.4. The results revealed a significant difference ($P < 0.05$) in peroxidase production by the organism when grown in the fermentation medium supplemented with different lignin model compounds as compared with the non-supplemented fermentation medium (LGO), which served as the control. The addition of the different lignin model compounds except guaiacol, which increased peroxidase production by over 100 % in *Bacillus* sp. FALADE-1 (LGO: 3.15 U mL^{-1}, LG + GA: 6.42 U mL^{-1}), repressed peroxidase production by the test strain. Our finding is comparable to a previous related study by Niladevi and Prema (2008) where 1 mmol L^{-1} pyrogallol and *p*-anisidine enhanced laccase production by 50 % in *Streptomyces psammoticus*. More so, Musengi *et al.* (2014) reported effective induction of peroxidase production by 0.1 mmol L^{-1} veratryl alcohol in *Streptomyces* sp. BSII#1. However, higher concentration of veratryl alcohol repressed peroxidase production in *Streptomyces* sp. BSII#1 (Musengi *et al.,* 2014).

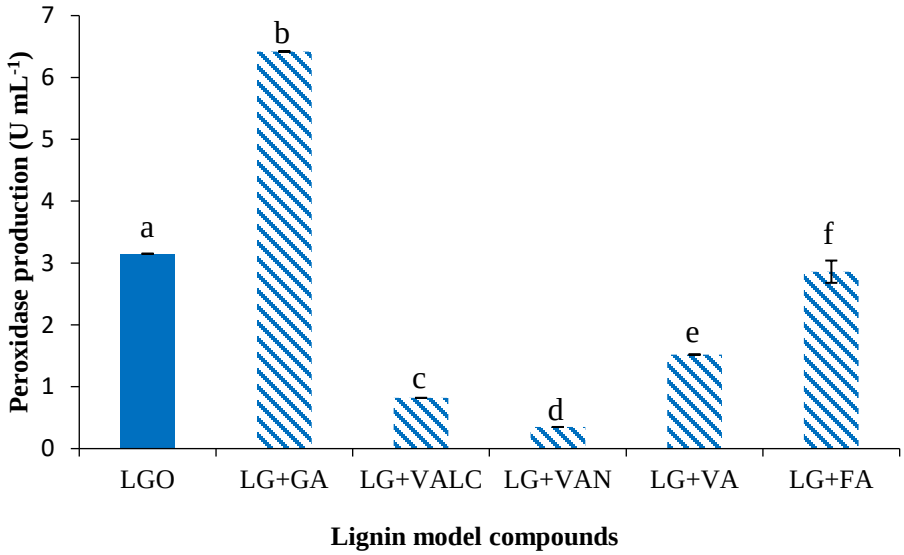

Fig. 5.4. Effect of lignin model compounds on peroxidase production by *Bacillus* sp. FALADE-1. Each column represent mean ± standard deviation. LGO: lignin only (control), LG+GA: lignin and guaiacol, LG+VALC: lignin and veratryl alcohol, LG+VAN: lignin and vanillin, LG+VA: lignin and vanillic acid, LG+FA: lignin and ferulic acid. Error bars with different alphabets are significantly different ($P < 0.05$).

5.3.5. Effect of nitrogen supplementations on peroxidase production

The effects of nature as well as concentration of nitrogen sources on the production of lignin modifying enzymes have been extensively studied (Kachlishvili *et al.*, 2005; Mikiashvili *et al.*, 2006; Stajic *et al.*, 2006). However, the findings from these studies are not usually consistent (Niladevi and Prema, 2008). In some cases, cultivation medium with adequate nitrogen enhanced ligninolytic enzyme production (Kaal *et al.*, 1995) while in others, production of ligninolytic enzymes was improved under nitrogen-limited conditions (Mester and Field, 1997; Gainfreda *et al.*, 1999; Galhaup *et al.*, 2002). Moreover, increase in nitrogen concentration may also limit the production of ligninolytic enzymes (Buswell, 1992). In this study, we evaluated the effect of supplementing the low organic nitrogen, yeast extract (0.1 g L^{-1}) in the fermentation medium with different inorganic nitrogen sources (5 g L^{-1}) including ammonium nitrate (AN), ammonium chloride (AC) and ammonium sulphate (AS). The results, as presented in Fig. 5.5 revealed a significant difference ($P < 0.05$) in peroxidase production by the test organism when cultivated in

the fermentation medium supplemented with inorganic nitrogen sources as compared with the control (fermentation medium without the supplements). Peroxidase production by *Bacillus* sp. FALADE-1 was enhanced by the addition of only ammonium sulphate (8.87 U mL^{-1}) while the other inorganic nitrogen sources studied seemed to repress peroxidase production by the organism (Fig. 5.5). This finding agrees with the result obtained by Kachlishvili *et al.* (2005) where manganese peroxidase production by some white-rot basidiomycetes was repressed by additional nitrogen, which was also corroborated by Mikiashvili *et al.* (2006) who reported that supplementation of defined fermentation medium with inorganic nitrogen sources decreased the production of lignin modifying enzymes including peroxidase.

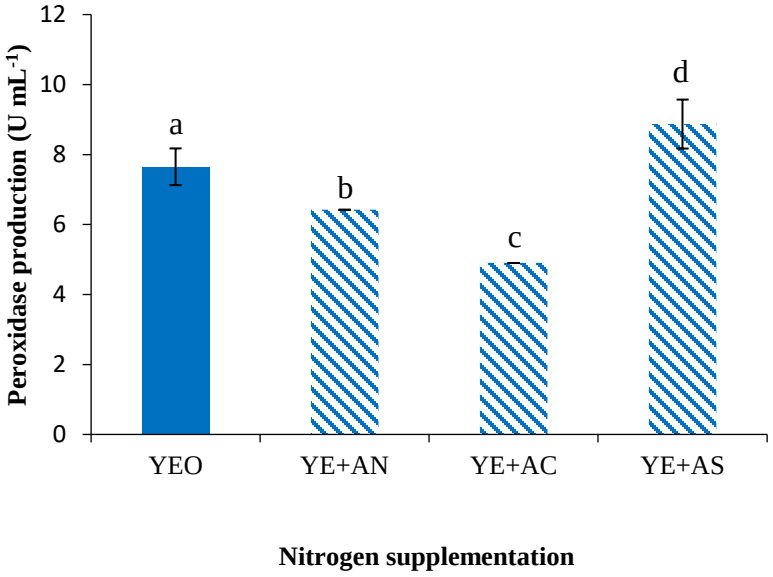

Fig. 5.5. Effect of nitrogen supplementation on peroxidase production by *Bacillus* sp. FALADE-1. Each column represent mean ± standard deviation. YEO: yeast extract only (control), YE+AN: yeast extract and ammonium nitrate, YE+AC: yeast extract and ammonium chloride, YE+AS: yeast extract and ammonium sulphate. Error bars with different alphabets are significantly different ($P < 0.05$).

5.3.6. Kinetics of enzyme production and bacterial growth

The kinetics of enzyme production by *Bacillus* sp. FALADE-1 and its cell growth pattern were assessed over a period of 120 h. The results, as presented in Fig. 5.6, showed that *Bacillus* sp. FALADE-1 attained its optimum peroxidase production at 48 h (late logarithmic growth phase), with specific peroxidase activity of 8.32 U mg^{-1} protein. This indicates that peroxidase production by the Bacillus strain was growth associated. However, the decline observed in the enzyme production by the organism after the optimal period of incubation, may perhaps, be as a result of denaturation or proteolysis (Fatokun *et al.,* 2017). This finding contradicts a number of previous related studies where the optimum peroxidase production was attained at 72 h of incubation (Rob *et al.,* 1997; Tuncer *et al.,* 1999; Nour El-Dein *et al.,* 2014).

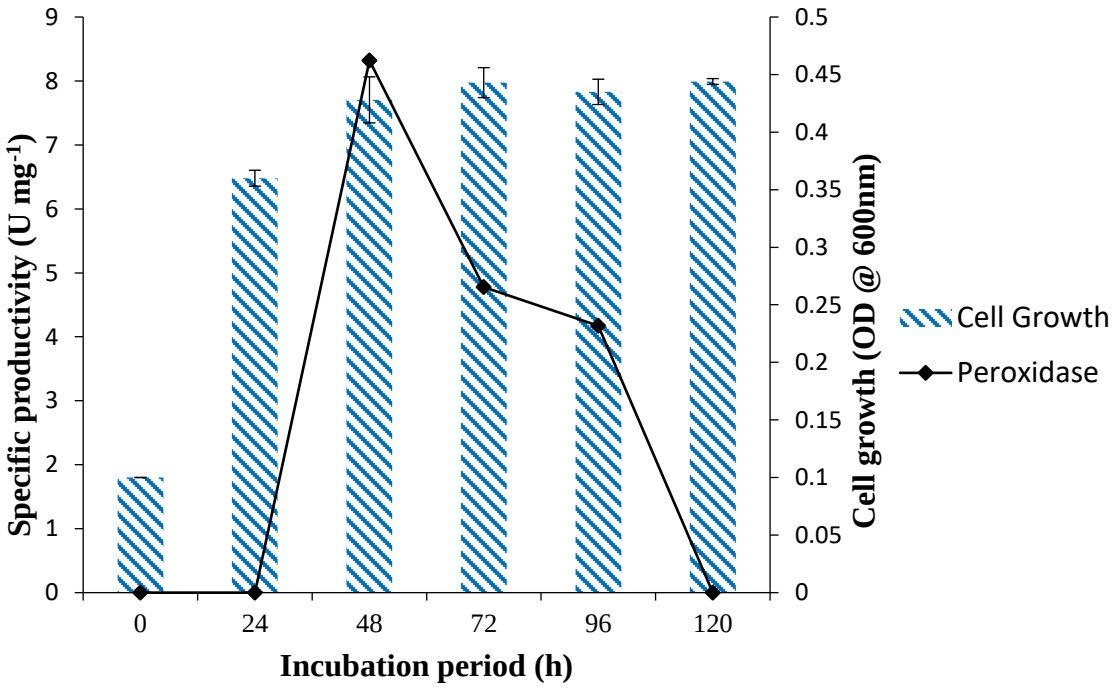

Fig. 5.6. Growth pattern and kinetics of peroxidase production by *Bacillus* sp. FALADE-1.

5.3.7. Detection of catalase-peroxidase gene (katG)

Fig. 5.7 showed the gel picture of the PCR amplified product. The band size of the amplified gene corresponded with the expected band size of 461bp. The blast result of the nucleotide sequence of

the amplified gene from *Bacillus* sp. FALADE-1 showed 100 % similarity to a catalase-peroxidase gene from *Sporotrichum thermophile* (PeroxiBase ID: 10141) when searched in PeroxiBase database (peroxibase.toulouse.inra.fr). More so, the deduced protein sequence formed a distinct cluster with bacterial catalase-peroxidases in the PeroxiBase (Fig. 5.8) and had an estimated molecular weight of 11.445 kDa with isoelectric point of 7.01. The nucleotide sequence was accessioned MF407314 and deposited in the GenBank of the NCBI as *Bacillus* sp. FALADE-1 catalase-peroxidase gene (BAF*katG*). The similarity of BAF*katG* to a fungal catalase-peroxidase gene (*katG*) might be as a result of horizontal gene transfer or evolution. This is corroborated by Passardi *et al.* (2007), in which the evolution of catalase-peroxidase genes in the PeroxiBase database was analyzed and observed that, occasionally, bacterial species that are closely related differ as they possess catalase-peroxidase genes of diverse origin or do not have any *katG*. Moreover, *katG* in eukaryotes (algae and fungi) have been suspected to originate from horizontal gene transfer of bacteria genome (Passardi *et al.*, 2007; Zamocky *et al.*, 2007).

Catalase-peroxidase (KatG) belongs to class I peroxidases of the peroxidase-catalase superfamily of heme-peroxidases (Zamocky and Obinger, 2010), also known as the superfamily of plant, bacterial and fungal heme-peroxidases (EC 1.11.1.7). The corresponding gene (*katG*) encodes a bifunctional enzyme with predominant catalase activity and significant peroxidase activity (Zamocky *et al.*, 2008) in an organism. Therefore, peroxidase activity expressed in *Bacillus* sp. FALADE-1 may be partly attributed to the presence of *katG* in the bacteria strain. Thus, overexpression of the gene could be further explored for large scale production of peroxidase as well as catalase through molecular optimization and genetic engineering, which has been problematic in fungi (Bugg *et al.*, 2011).

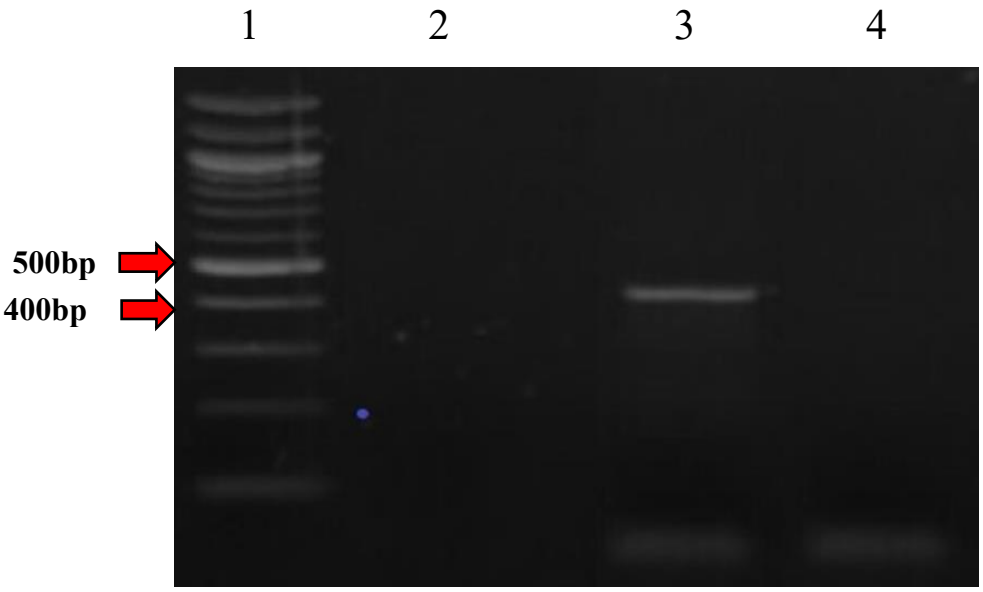

Fig. 5.7. Gel picture of the PCR amplified peroxidase gene. Lane 1: 100bp DNA ladder, lane 2: Empty, lane 3: PCR amplified product from *Bacillus* sp. FALADE-1, lane 4: Negative control.

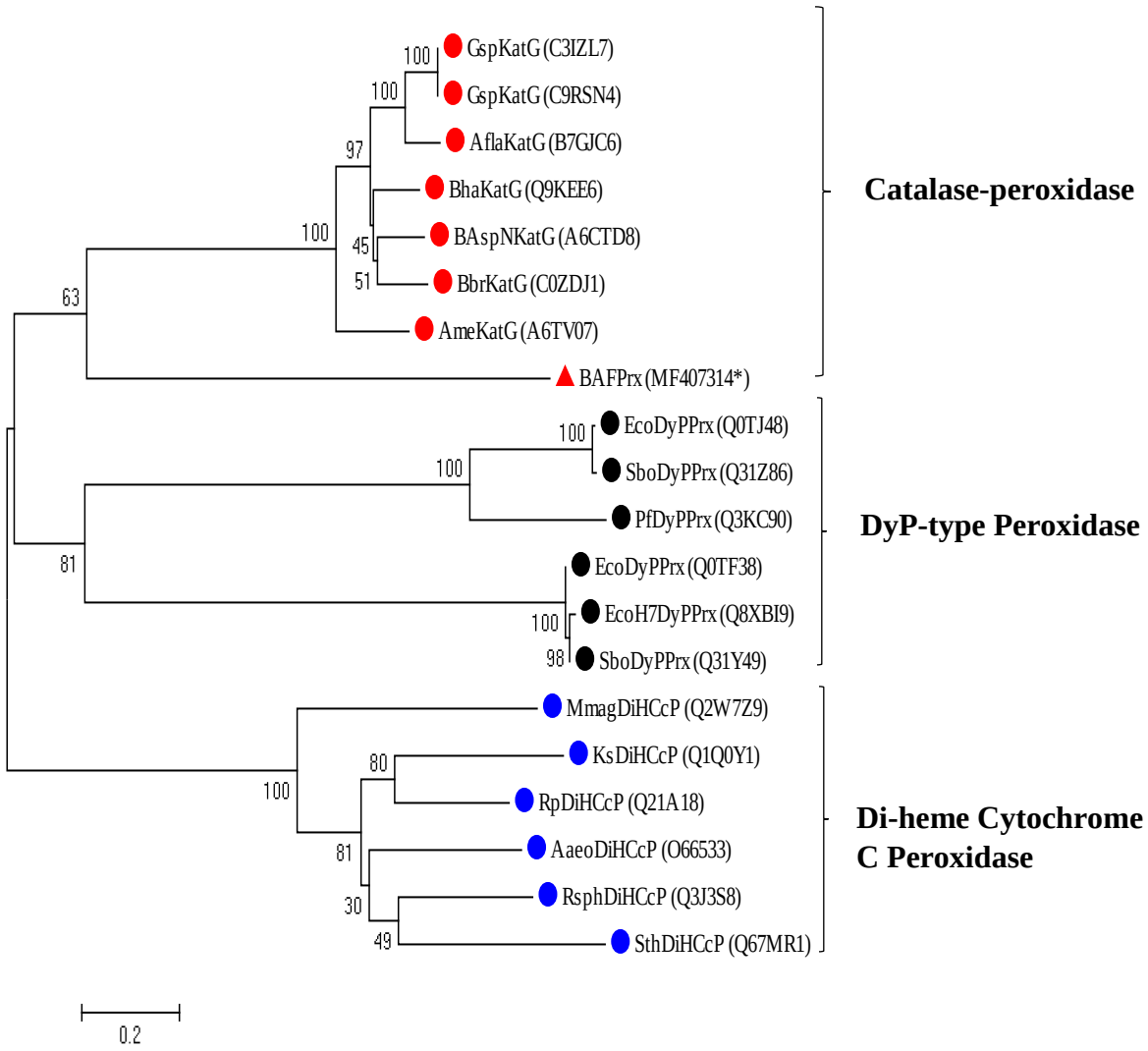

Fig. 5.8. Phylogenetic tree showing the family of *Bacillus* sp. FALADE-1 peroxidase in the bacterial heme-peroxidases from PeroxiBase. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Red tips indicate Catalase-peroxidase, black tips indicate DyP-type Peroxidase while the blue tips represent Di-heme Cytochrome C Peroxidase (DiHCcP). The red triangular tip indicates the studied protein sequence (BAFPrx). The UniProtKB reference numbers/GenBank accession number* of the proteins are indicated in parentheses.

Afla: *Anoxybacillus flavithermus*, Ame: *Alkaliphilus metalliredigenes*, BAspN: *Bacillus* sp. NRRL B-14911, Bbr: *Brevibacillus brevis*, Bha: *Bacillus halodurans*, Gsp: *Geobacillus* sp., Aaeo: *Aquifex aeolicus*, Ks: *Kuenenia stuttgartiensis*, Mmag: *Magnetospirillum magneticum*, Rp: *Rhodopseudomonas palustris*, Rsph: *Rhodobacter sphaeroides*, Sth: *Symbiobacterium thermophilum*, Eco: *Escherichia coli*, Pf: *Pseudomonas fluorescens*, Sbo: *Shigella boydii*, EcoH7: *Escherichia coli* 0157:H7, BAFPrx: *Bacillus* sp. FALADE-1 Peroxidase, KatG: Catalase-peroxide, DyPPrx: Dye Decolourizing Peroxidase.

5.4. Conclusion

Peroxidase production by *Bacillus* sp. FALADE-1, isolated from Hogsback forest reserve in the Eastern Cape, South Africa increased by 2.22-fold as the gene encoding a bifunctional enzyme activity was detected in the bacteria strain. This indicates the dexterity of the organism for large scale peroxidase production capable of meeting the increasing industrial demand for peroxidase. It also represents a potential source of catalase that can be exploited for biotechnological applications. Nonetheless, the identification of *katG* in *Bacillus* sp. FALADE-1 prompts the need for further study in molecular optimization and genetic engineering.

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CHAPTER SIX

Optimization of process parameters for exoperoxidase production by *Ensifer adhaerens* NWODO-2 and PCR detection of catalase-peroxidase gene (*KatG*)

(*Submitted to Applied and Environmental Microbiology*)

Abstract

Given the high utility of peroxidase in several industrial processes, the search for novel microorganisms with enhanced peroxidase production capacity is of keen interest. This study investigated the process conditions for optimum exoperoxidase production by *Ensifer adhaerens* NWODO-2 (KX640918), a new ligninolytic proteobacteria with peroxidase production potential. Also, some agricultural residues were valorized for exoperoxidase production under solid state fermentation. Subsequently, the gene coding for peroxidase activity was detected through polymerase chain reaction (PCR) and Sanger dideoxy sequencing technology. Peroxidase production was optimum at an initial medium pH 7 (5.83 ± 0.00 U mL^{-1}), incubation temperature of 30 ^{o}C (5.83 ± 0.00 U mL^{-1}) and agitation speed of 100 rpm (5.31 ± 1.23 U mL^{-1}) using alkali lignin fermentation medium supplemented with guaiacol as the most effective inducer and ammonium sulphate as the best inorganic nitrogen. Optimum peroxidase production by *Ensifer adhaerens* NWODO-2 was attained at 48 h with specific productivity of 12.76 ± 1.09 U mg^{-1}. Interestingly, probable laccase production was observed with optimum specific productivity of 12.76 ± 0.45 U mg^{-1} at 72 h. The highest exoperoxidase yield (37.50 ± 0.00 U mg^{-1}) was observed with sawdust as substrate under solid state fermentation. Although, the blast search of the nucleotide sequence of the amplified gene in UniProtKB showed 70.5% similarity to *Ensifer adhaerens* uncharacterized protein, phylogenetic analysis suggests the gene may encode a catalase-peroxidase with an estimated molecular weight of 31.145 kDa and isoelectric point of 11.47. Then, the sequence was deposited in the GenBank as *Ensifer adhaerens* NWODO-2 catalase-peroxidase gene (*katG*) under the accession number MF374336. Our findings suggest that *katG* may be responsible for the peroxidase activity expressed in *Ensifer adhaerens* NWODO-2.

Importance

The increased industrial application potentials of peroxidase have led to high market demand which could not be met by only Horseradish Peroxidase (HRP), the major commercially available peroxidase. Consequently, the need for alternative and efficient peroxidase producers is imperative. Over the years, fungi, predominantly, white rot basidiomycetes, have been the most efficient producers of peroxidase. However, optimization of fungal enzyme through genetic engineering is difficult, hence the imperativeness of peroxidase-producing bacteria with promising potential for molecular optimization. The significance of our research is in detecting the gene encoding peroxidase activity in *Ensifer adhaerens* NWODO-2, which could further be explored for enhanced peroxidase production through gene cloning and overexpression.

Keywords: Bifunctional enzyme, catalase-peroxidase, enzyme production, peroxidase, polymerase chain reaction, proteobacteria.

6.1. Introduction

Peroxidases are oxidative enzymes with the capacity to oxidize a wide range of organic and inorganic compounds using hydrogen peroxide as electron acceptor. They are characterized by several biotechnological potentials spanning through different industries including textile, paper and pulp, chemical, water and cosmetics (Draelos, 2015; Taboada-Puig *et al.,* 2015).

Specifically, the potential of peroxidases for development of biosensors and diagnostic kits has been reported (Jia *et al.,* 2002; Agostini *et al.,* 2002). Some of the recent applications of peroxidases include the use of lignin peroxidase as an alternative to hydroquinone in the development of skin-lightening agents and the application of versatile peroxidase for removal of endocrine disrupting chemicals (EDCs) in wastewater (Draelos, 2015; Taboada-Puig *et al.,* 2015; Falade *et al.,* 2017a). Among other peroxidase applications are biopulping and biobleaching (Hatakka *et al.,* 2003), degradation of textile dyes (Kalyani *et al.,* 2011) and synthesis of natural aromatic flavours (Barbosa *et al.,* 2008). However, the industrial application of peroxidases is limited by high cost of production, inadequate amount of enzyme produced (Ferrer *et al.,* 1991; Torres *et al.,* 2003) and enzyme instability in the presence of high concentrations of hydrogen peroxide (Valderrama *et al.,* 2002).

Given the diverse application potentials of peroxidases, increased demand is inevitable. This has therefore necessitated the search for organisms with enhanced peroxidase production capacity. Over the years, fungi have been the most efficient producers of exoperoxidases (Ikehata *et al.,* 2004; Urek and Pazarlioglu, 2007; Hariharan and Nambisan, 2013). Nevertheless, bacteria, largely actinomycetes, have also shown potential for exoperoxidase production (Mercer *et al.,* 1996; Tuncer *et al.,* 2004; 2009; Musengi *et al.,* 2014). Peroxidases are also found in other living organisms but occur intracellularly and are produced in minute quantity. Exoproduction of peroxidase is advantageous over the intracellular production as the purification and recovery of an exoenzyme is simplified while the isolation and purification of an intracellular enzyme is complex and time-consuming (Musengi *et al.,* 2014).

Ensifer adhaerens NWODO-2 (KX640918) is a new lignin-degrading bacteria (Falade *et al.,* 2017b) belonging to the class: alpha-proteobacteria, one of the few classes of bacteria with a record of lignin degradation activities (Bugg *et al.,* 2011; Taylor *et al.,* 2012). The whole genome sequencing of *Rhizobium* sp. strain YS-1r, a lignin degrading alpha proteobacteria revealed the

presence of a gene encoding the expression of DyP-type peroxidase (Prabhakaran *et al.,* 2015), an extracellular lignin modifying enzyme. More so, gene prediction analysis revealed high expression of 6 putative genes coding for peroxidase in *Klebsiella* sp. strain BRL 6-2 (Woo *et al.,* 2015), a ligninolytic gamma proteobacteria. Furthermore, production of peroxidase by another proteobacteria, Pseudomonas species, has been reported (Kalyani *et al.,* 2011). It is clear that proteobacteria is an emerging ligninolytic bacteria with enormous potential for the production of lignin modifying enzymes including peroxidases. Our preliminary screening has revealed the peroxidase production potential of *E. adhaerens* NWODO-2 (Falade *et al.,* 2017b). This study therefore seeks to optimize the exoperoxidase production by *E. adhaerens* NWODO-2 and detect the gene encoding the expressed peroxidase activity in the organism.

6.2. Materials and methods

6.2.1. Isolation, screening and molecular identification

The organism was isolated from a water sample collected from Tyhume River in Alice, South Africa by enrichment technique using alkali lignin (Sigma-Aldrich, SA) as the sole carbon source (Sasikumar *et al.,* 2014). Thereafter, the organism was selected based on its ligninolytic and peroxidase production potential (Falade *et al.,* 2017b). The 16S rRNA gene sequence analysis showed that the bacteria strain had 99% similarity to *E. adhaerens* S4-6 (KY496256) as reported elsewhere (Falade *et al.,* 2017b).

6.2.2. Exoperoxidase production under submerged fermentation (SMF)

Peroxidase was produced using submerged fermentation as described by Falade *et al.* (2017b). The culture was incubated at 30 ^{o}C and 140 rpm for 48 h. Thereafter, the crude enzyme was prepared as reported elsewhere (Falade *et al.,* 2017b) and the recovered supernatant was utilized as crude enzyme for peroxidase activity assay.

6.2.3. Peroxidase activity assay

The peroxidase activity was evaluated by the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin using the modified method of Chance and Maehly (1955) as described by Falade *et al.* (2017b).

6.2.4. Determination of process parameters for optimum exoperoxidase production

The process conditions for optimum exoperoxidase production were determined as described by Fatokun *et al.* (2016). Briefly, the various culture parameters were adjusted for optimum peroxidase production using the conventional method of one factor at a time. Then the determined optimized conditions were used in subsequent fermentation (Fatokun *et al.,* 2016). The optimum initial medium pH for exoperoxidase production was determined by growing *E. adhaerens* NWODO-2 in an alkali lignin fermentation medium with the initial pH ranging from 3 to 11 at 30 ^{o}C. Thereafter, the optimum incubation temperature was determined by growing the test bacteria at varying temperature range of 20 to 45 ^{o}C at 5 ^{o}C interval in the same fermentation medium with pH 7, being the optimum initial medium pH for the enzyme production. Then, the effect of agitation speed on exoperoxidase production was assessed by growing the test organism in the fermentation medium at its optimum temperature (30 ^{o}C) and initial pH (pH 7) at varying agitation speeds ranging from static condition to 200 rpm at 50 rpm intervals.

6.2.5. Effect of nutrient compositions on exoperoxidase production

The effect of lignin monomers on exoperoxidase production was assessed by growing *E. adhaerens* NWODO-2 in an alkali lignin fermentation medium supplemented with 1 mmol L^{-1} of different lignin monomers: guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid (Musengi *et al.,* 2014) using the following optimum parameters for the enzyme production: temperature (30 ^{o}C), initial pH (pH 7) and agitation speed (100 rpm). Subsequently, the test organism was grown in an alkali lignin fermentation medium wherein yeast extract was supplemented with different inorganic nitrogen sources (ammonium nitrate, ammonium chloride and ammonium sulphate) at the optimum temperature (30 ^{o}C), initial pH (pH 7) and agitation speed (100 rpm) for exoperoxidase production, with guaiacol (1 mmol L^{-1}) being used as an inducer.

6.2.6. Time course assay

The exoperoxidase production by *E. adhaerens* NWODO-2 as well as its growth kinetics were assessed under optimized process conditions for 144 h as described by Tuncer *et al.* (1999) with slight modifications. The culture was intermittently withdrawn at 24 h interval (Tuncer *et al.,* 1999) and assayed for exoperoxidase and non-peroxide dependent exoenzyme production, as well as total

protein concentration. Also, the cell growth was monitored by measuring the optical density (OD) of the culture at 600 nm.

6.2.7. Determination of protein concentration

The total protein concentration was determined using Bradford method (Bradford, 1976). Briefly, 250 µL of Bradford reagent was added to 10 µL of the supernatant in a 96-well microtitre plate while 10 µL of distilled water was used in place of the sample in the blank. The mixture was subsequently incubated at room temperature for 15 min. Thereafter, the absorbance was measured at 595 nm using SynergyMx 96-well microtitre plate reader (BioTeK Instruments, USA). The protein concentration was extrapolated from the standard curve constructed using bovine serum albumin (BSA). The specific enzyme productivity was expressed as U mg^{-1} protein.

6.2.8. Substrate preparation for solid state fermentation (SSF)

Some agricultural residues including sawdust, wheat straw and corn stover were valorized for exoperoxidase production under SSF. The sawdust was obtained from a sawmill in Alice metropolis, South Africa while the wheat straw and corn stover were obtained from the agricultural research farm of the University of Fort Hare in Alice, Eastern Cape, South Africa. Thereafter the residues were washed, air-dried and ground with a laboratory milling machine (Lasec, SA). The milled products were thereafter sieved into powder form (with size less than 500 µm). The processed agricultural residues were subsequently used as solid substrates for exoperoxidase production.

6.2.9. Exoperoxidase production under SSF

Peroxidase was produced under SSF using the modified method of Neifar *et al.* (2016). Briefly, 5 g of substrate was weighed into 100 mL conical flasks and dampened with 10 - 20 mL of minimal salt medium (4.55 g L^{-1} K_2HPO_4, 0.53 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 5 g L^{-1} $(NH_4)_2SO_4$, 0.1 g L^{-1} yeast extract) modified with 1 mmol L^{-1} of guaiacol, with an initial pH of 7, being the optimum initial pH for peroxidase production by the strain under investigation. Then, the preparations in the flasks were autoclaved at 121 ^oC for 15 min and thereafter inoculated with 1 mL of standard inoculum of the bacterial suspension in normal saline (O.D. 600 nm ≈ 1.0). After 48 h incubation (the time for optimum peroxidase production by *E. adhaerens* NWODO-2), 20 mL of 0.1 mol L^{-1}

potassium phosphate buffer (pH 6) was added to the flasks and stirred for 30 min for the extraction of crude enzyme. Subsequently, the contents of the flasks were centrifuged at 10000 x g for 10 min at 4 ^{o}C using benchtop centrifuge (SIGMA 1-14K, Lasec, SA), and the recovered supernatant was used as crude enzyme for peroxidase assay.

6.2.10. PCR detection of peroxidase encoding gene

DNA was extracted from the pure culture of *E. adhaerens* NWODO-2 using boiling method (Maugeri *et al.*, 2006) as follows: few colonies of the organism were suspended in 200 µL of nuclease-free water and boiled for 10 min in a Dri-Block DB-3D (TECHNE, Lasec, SA) set at 100 ^{o}C, followed by centrifugation at 20000 x g for 5 min (HERMLE Z 233 M-2, Lasec, SA). The supernatant was subsequently used as DNA template for polymerase chain reaction (PCR), which was performed with the following newly designed primers, synthesized by Inqaba Biotech, South Africa: (Forward: 5' CGACCCTGCCTACGAAAAGAT 3') and (Reverse: 5' ATAGTTGCGGAAGCCCTCGGA 3') in a PCR thermocycler apparatus (G-STORM, UK) using the optimized conditions of initial denaturation at 95 ^{o}C (5min), denaturation at 94 ^{o}C (1min), annealing at 58 ^{o}C (1 min), extension at 72 ^{o}C (1 min) for 35 cycles and final extension at 72 ^{o}C (5 min). The total reaction volume was 25 µL, which was composed of 12.5 µL of master mix (BioLabs, SA), 1µL each of both forward and reverse primers, 5.5 µL of nuclease-free water and 5 µL of the DNA template. The amplified product was subjected to electrophoresis in 1.5 % agarose gel (Merck, SA) and visualized in ethidium bromide (Sigma-Aldrich, SA) staining using ultraviolet trans-illuminator (Alliance 4.7, France). Thereafter, the PCR product was analyzed using Sanger dideoxy sequencing method followed by a BLAST search in UniProt Knowledgebase and PeroxiBase database (peroxibase.toulouse.inra.fr) using Blastx program.

6.2.11. Phylogenetic analysis

Phylogenetic analysis of the deduced protein sequence from *E. adhaerens* NWODO-2 and selected bacterial peroxidases in the PeroxiBase was conducted using neighbour-joining method (Saitou and Nei, 1987) in MEGA 7.0 software (Kumar *et al.*, 2016) while the physicochemical properties (molecular weight and isoelectric point) were determined by geneious 10.2.2.

6.2.12. Data analysis

Results of replicates were expressed as mean ± standard deviation (STD) using Microsoft Excel Spreadsheet. Data were subsequently subjected to one-way ANOVA using GraphPad Prism 7 followed by Tukey's Multiple Comparison Test. Significance was accepted at $P \leq 0.05$.

6.2.13. Accession number

The nucleotide sequence data reported in this study is available in the GenBank nucleotide sequence database under accession number MF374336 (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/nucleotide).

6.3. Results

6.3.1. Effect of initial medium pH on exoperoxidase production

The effect of initial medium pH on exoperoxidase production is presented in Fig. 6.1. The results showed that *E. adhaerens* NWODO-2 produced peroxidase over a pH range of 5 to 9 with the optimum production observed at pH 7 (5.83 ± 0.00 U mL^{-1}) while no peroxidase activity was detected at pH 3, 4, 10 and 11. The results revealed a significance difference *($P < 0.05$)* in peroxidase production across the pH but there was no significant difference *($P > 0.05$)* in peroxidase production at pH 6 (5.02 ± 0.00 U mL^{-1}) and pH 7 (5.83 ± 0.00 U mL^{-1}).

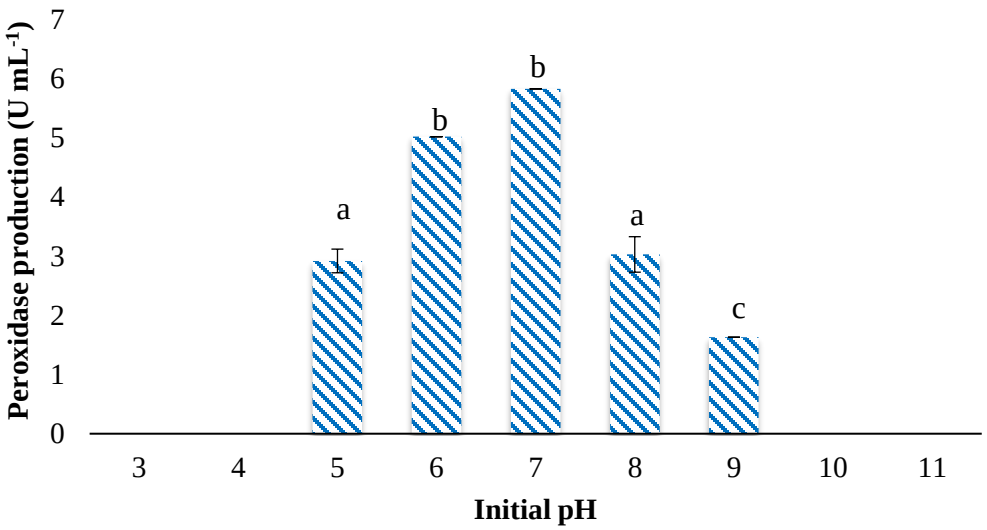

Fig. 6.1. Effect of initial medium pH on exoperoxidase production by *E. adhaerens* NWODO-2 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P> 0.05*).

6.3.2. Effect of incubation temperature on exoperoxidase production

The effect of incubation temperature on exoperoxidase production by *E. adhaerens* NWODO-2 is presented in Fig. 6.2. The results revealed a significant difference *(P < 0.05)* in peroxidase production across the temperatures: 20 – 45 ^{o}C with optimum peroxidase production observed at 30^{o}C (5.83 ± 0.00 U mL^{-1}). Nonetheless, post hoc comparison test revealed no significant difference *(P > 0.05)* in peroxidase production at 20 ^{o}C (1.05 ± 0.00 U mL^{-1}), 35 ^{o}C (0.58 ± 0.00 U mL^{-1}), 40 ^{o}C (1.52 ± 0.59 U mL^{-1}) and 45 ^{o}C (0.94 ± 0.12 U mL^{-1}).

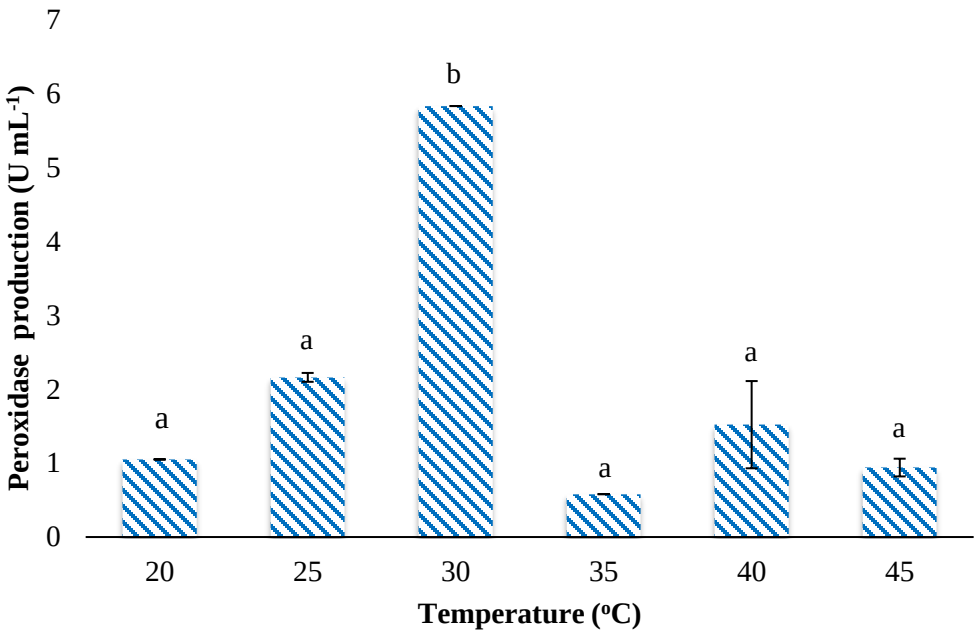

Fig. 6.2. Effect of incubation temperature on exoperoxidase production by *E. adhaerens* NWODO-2 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P> 0.05*).

6.3.3. Effect of agitation speed on exoperoxidase production

The results on effect of agitation speed on exoperoxidase production by *E. adhaerens* NWODO-2 is presented in Fig. 6.3. The results showed a significant difference *(P < 0.05)* in peroxidase production under static condition, and across all agitation speeds (50-200 rpm) with optimum peroxidase production observed at 100 rpm (5.31 ± 1.23 U mL^{-1}). However, post hoc comparison test revealed no significant difference *(P > 0.05)* in the peroxidase production at 150 rpm (3.50 ± 0.12 U mL^{-1}) and 200 rpm (2.69 ± 0.12 U mL^{-1}).

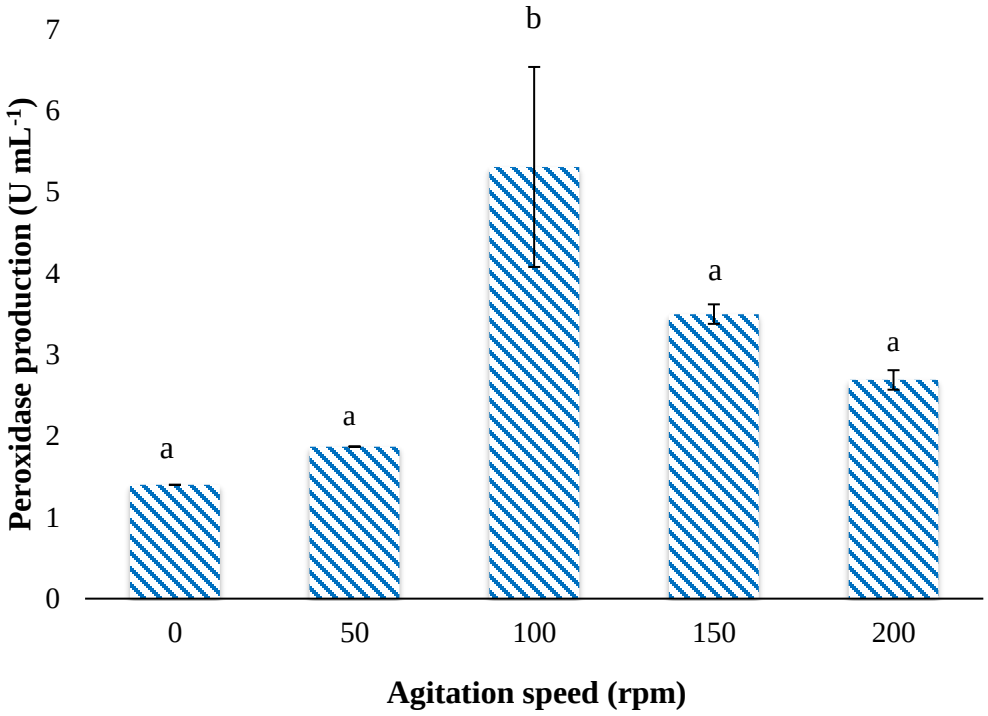

Fig. 6.3. Effect of agitation rate on exoperoxidase production by *E. adhaerens* NWODO-2 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P> 0.05*).

6.3.4. Effect of lignin monomers on exoperoxidase production

The effect of supplementing the fermentation medium with 1 mmol L^{-1} of various lignin monomers (Guaiacol-GA, Veratryl alcohol-VALC, vanillin-VAN, vanillic acid-VA and ferullic acid-FA) on exoperoxidase production is shown in Fig. 6.4. The results showed a significant increase *(P < 0.05)* in peroxidase production by *E. adhaerens* NWODO-2 grown in fermentation medium supplemented with guaiacol: KL+GA (5.25 ± 0.00 U mL^{-1}) when compared with non-supplemented fermentation medium (KL), which serves as the control (2.10 ± 0.00 U mL^{-1}). However, there was no significant difference in peroxidase production when supplemented with other lignin monomers.

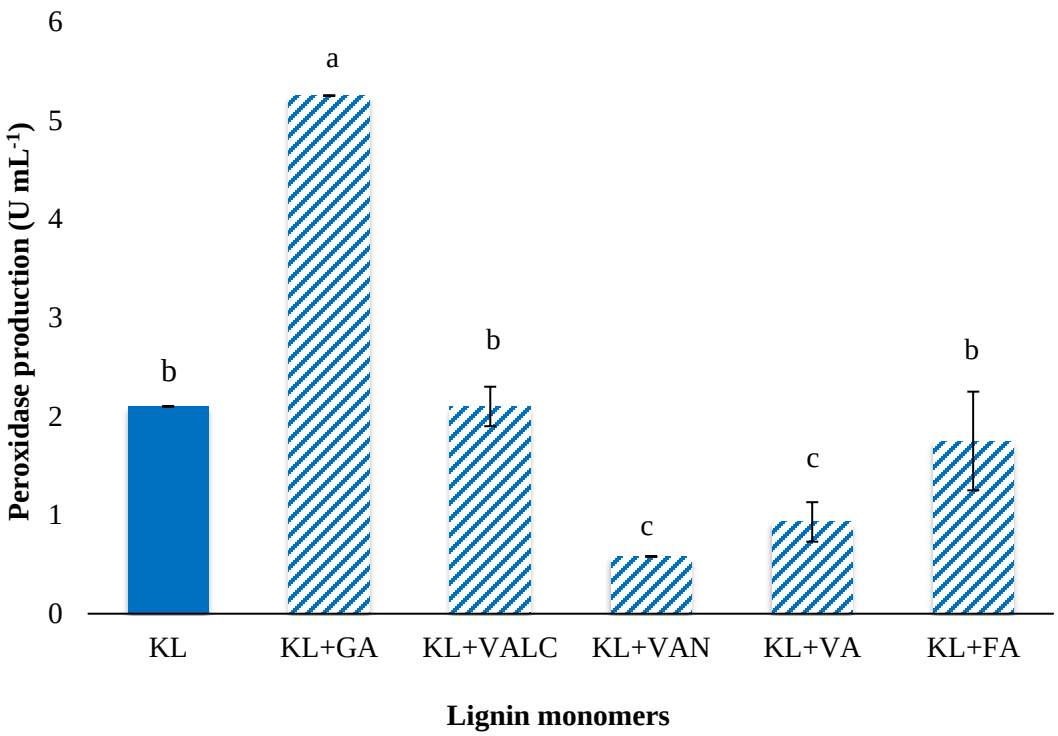

Fig. 6. 4. Effect of lignin monomers on exoperoxidase production by *E. adhaerens* NWODO-2 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P > 0.05*). KL: kraft lignin (Control), GA: guaiacol, VALC: veratryl alcohol, VAN: vanillin, VA: vanillic acid, FA: ferullic acid.

6.3.5. Effect of nitrogen supplementations on exoperoxidase production

The effect of supplementing yeast extract with different inorganic nitrogen sources in the fermentation medium is presented in Fig. 6.5. The results showed a significant increase *(P < 0.05)* in peroxidase production by *E. adhaerens* NWODO-2 grown in the fermentation medium supplemented with inorganic nitrogen sources (ammonium nitrate; ammonium chloride; and ammonium sulphate) with ammonium sulphate (YE + Ammonium Sulphate) giving the maximum peroxidase yield (11.31 ± 0.12 U mL^{-1}) when compared with non-supplemented fermentation medium, YE only (4.67 ± 0.00 U mL^{-1}).

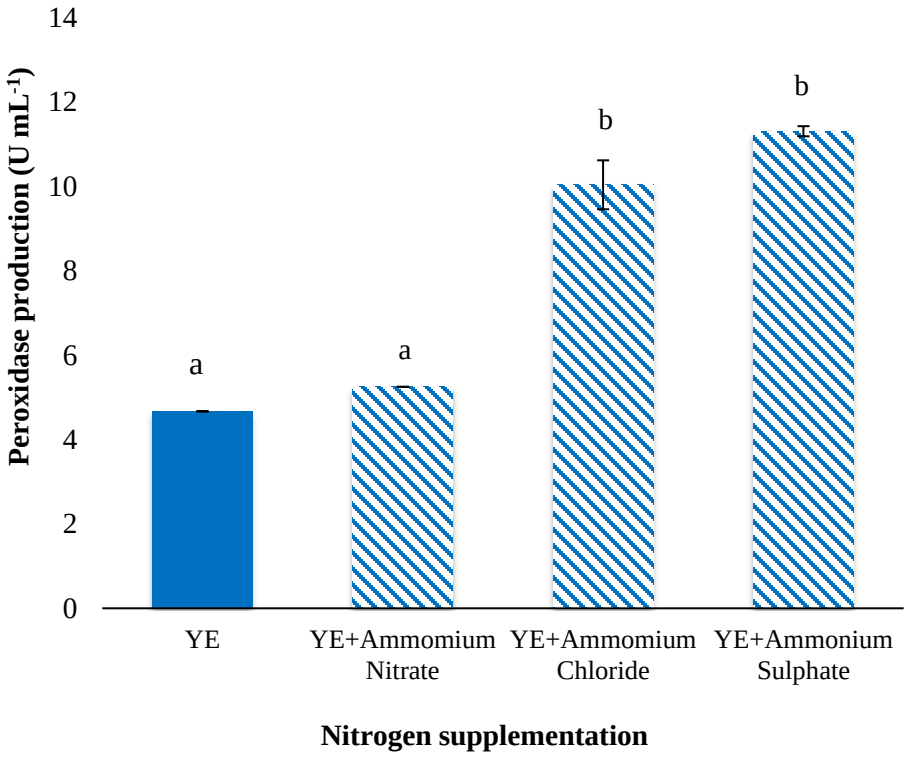

Fig. 6.5. Effect of nitrogen supplementations on exoperoxidase production by *E. adhaerens* NWODO-2 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P> 0.05*). YE: Yeast Extract.

6.3.6. Exoperoxidase production over a time course

The *Ensifer* strain was evaluated for extracellular enzyme production in the presence and absence of hydrogen peroxide over a period of 144 h and the results are presented in Fig. 6.6. The results indicated that *E. adhaerens* NWODO-2 attained optimum peroxidase production (12.19 ± 1.05 U mL^{-1}) at 48 h, corresponding to the late logarithmic growth phase with specific productivity of 12.76 ± 1.09 U mg^{-1} (Fig. 6a, b). However, there was a significant decrease in exoperoxidase production as from 72 h, which corresponds to the early stationary growth phase. More so, extracellular enzyme activity (23.10 ± 0.82 U mL^{-1}) was detected in the absence of hydrogen peroxide and was optimum at 72 h with specific activity of 12.76 ± 0.45 U mg^{-1} protein (Fig. 6a, b).

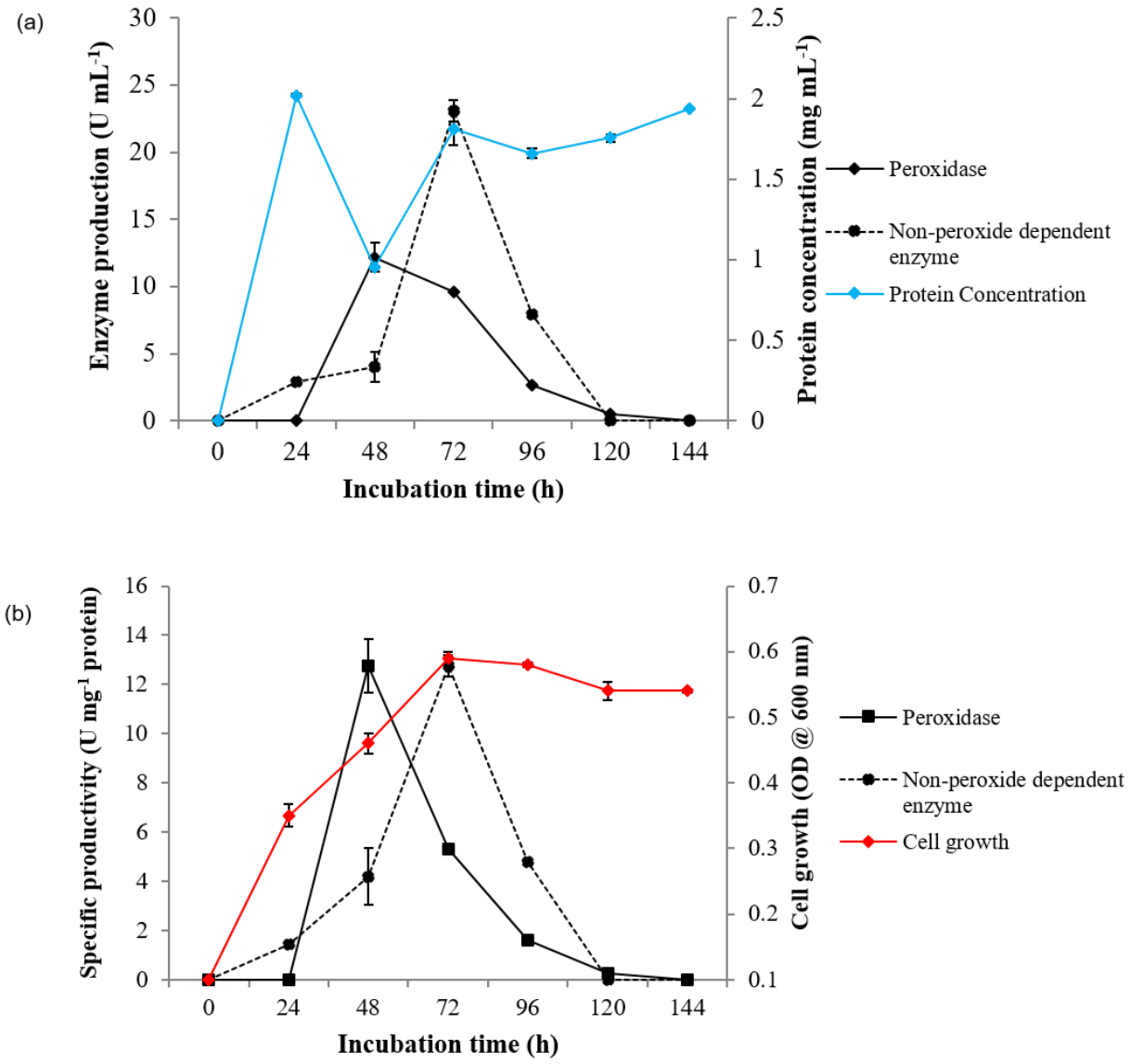

Fig. 6.6. Time course of extracellular enzyme production by *E. adhaerens* NWODO-2.
(a). Enzyme production and protein concentration (b). Specific productivity and cell growth.

6.3.7. Valorization of agricultural residues for exoperoxidase production

The results on valorization of agricultural residues for exoperoxidase production by *E. adhaerens* NWODO-2 under SSF is presented in Table 6.1. The results showed a significant difference *($P < 0.05$)* in peroxidase production by the test organism when grown on the selected substrates, with sawdust having the highest yield (37.50 ± 0.00 U mg^{-1}) while the lowest yield was observed on corn stover (3.76 ± 0.00 U mg^{-1}). However, enzyme activity was not detected in the absence of hydrogen peroxide when grown on sawdust and wheat straw but a specific extracellular enzyme activity of 1.23 ± 0.21 U mg^{-1} was detected in the absence of hydrogen peroxide when grown on corn stover.

Table 6.1. Valorization of some agricultural residues for exoperoxidase production by *E. adhaerens* NWODO-2 under SSF.

Agricultural residue	**Protein concentration (mg mL^{-1})**	**Enzyme assay (with H_2O_2)**		**Enzyme assay (without H_2O_2)**	
		Peroxidase production (U mL^{-1})	Specific productivity (U mg^{-1})	Probable laccase production (U mL^{-1})	Specific productivity (U mg^{-1})
Sawdust	0.028 ± 0.0^{a}	1.05 ± 0.00^{a}	37.50 ± 0.00^{a}	NAD*	NAD*
Wheat Straw	1.023 ± 0.116^{b}	5.37 ± 0.00^{b}	5.25 ± 0.00^{b}	NAD*	NAD*
Corn Stover	1.366 ± 0.021^{b}	5.13 ± 0.00^{b}	3.76 ± 0.00^{c}	1.93 ± 0.53	1.23 ± 0.21

Values represent mean ± standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different (P> 0.05). SSF: solid state fermentation. NAD*– No Activity Detected.

6.3.8. PCR detection of catalase-peroxidase gene

Fig. 6.7 shows the gel picture of the PCR amplified product from *E. adhaeren* NWODO-2. The results showed the band size of the amplified gene as 800 bp.

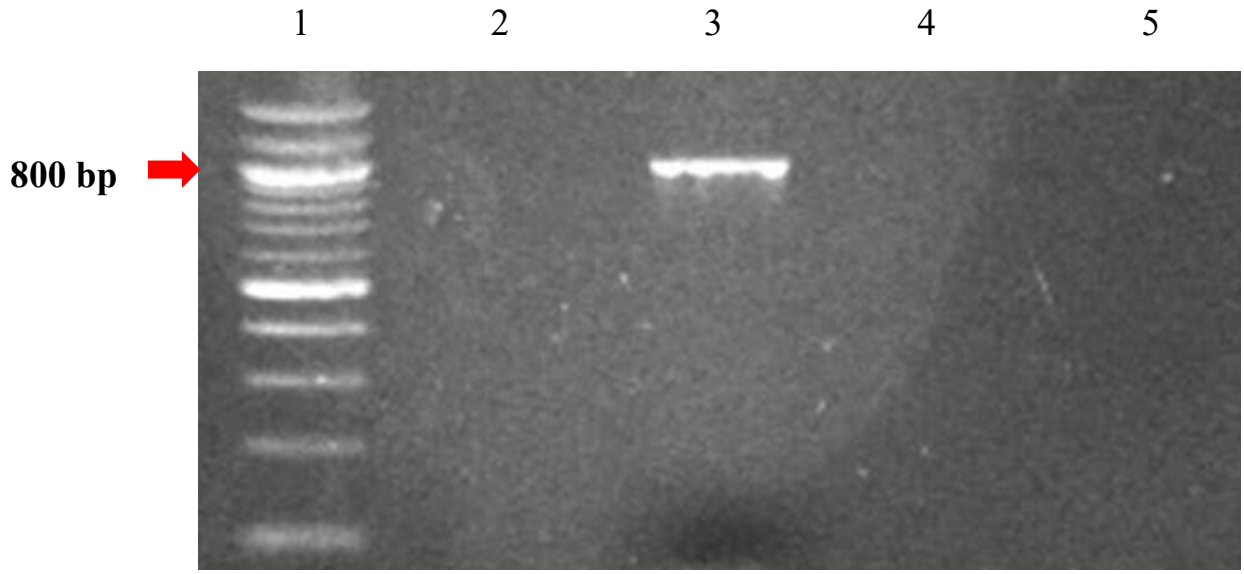

Fig. 6.7. Gel picture of the PCR amplified product. Lane 1: 100bp DNA ladder, lane 2: Empty, lane 3: PCR amplified product from *E. adhaerens* NWODO-2, lane 4: Negative control.

The blast search of the nucleotide sequence in the UniProt Knowledgebase using blastx program showed 70.5% similarity to *E. adhaerens* uncharacterized protein (ANK75658).

However, phylogenetic analysis of the expressed protein sequence and selected bacterial heme peroxidases in the PeroxiBase database (peroxibase.toulouse.inra.fr) showed that it clustered with catalase-peroxidases (Fig. 6.8). This finding suggests that the amplified gene from *E. adhaerens* NWODO-2 may encode a novel catalase-peroxidase with an estimated molecular weight of 31.145 kDa and isoelectric point of 11.47. The nucleotide sequence was then deposited in the GenBank under the accession number MF374336.

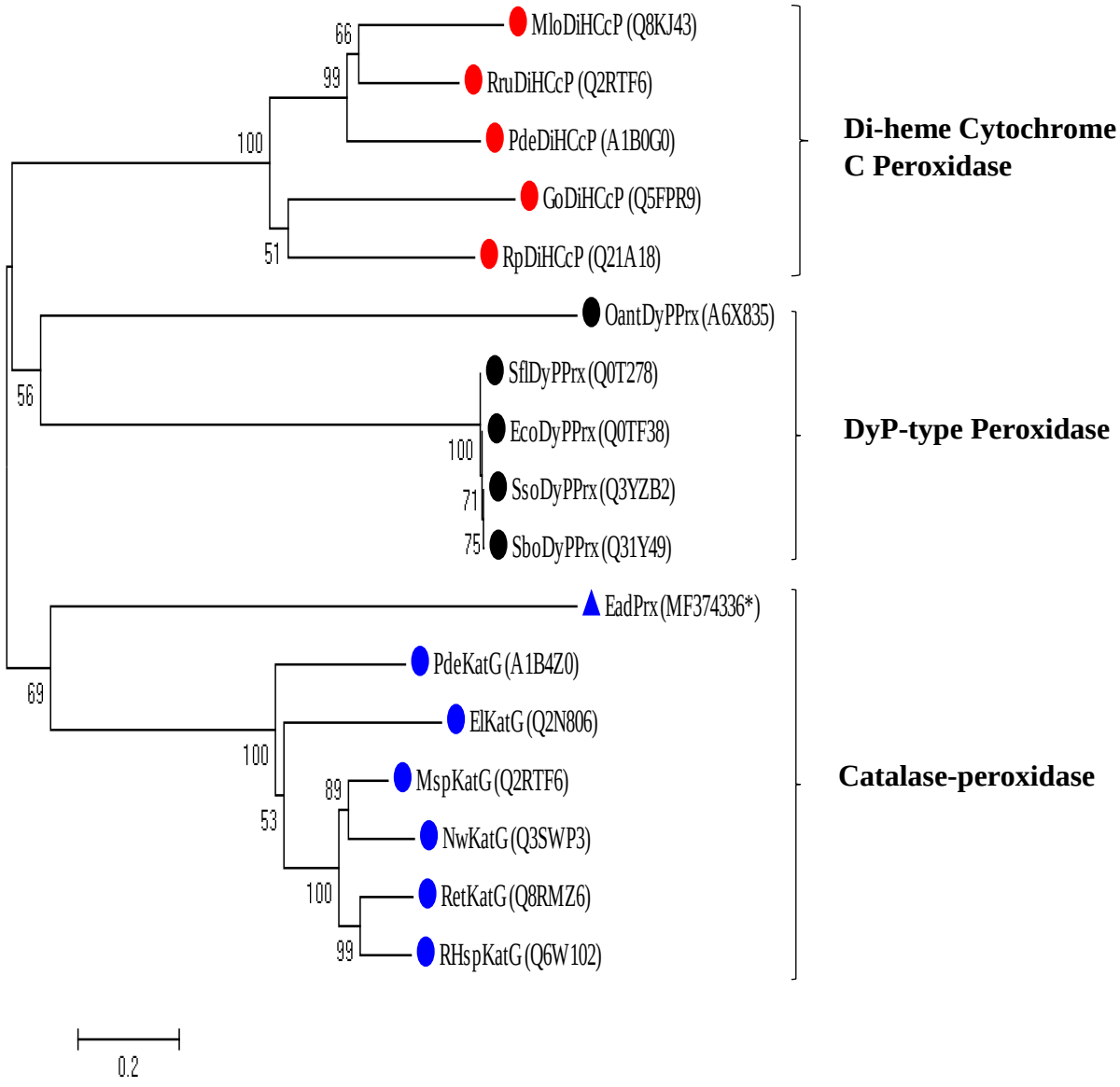

Fig. 6.8. Unrooted phylogenetic tree showing the relationship of *E. adhaerens* NWODO-2 peroxidase with selected bacterial heme-peroxidases in the PeroxiBase. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Red tips indicate Di-heme Cytochrome C Peroxidase (DiHCcP) family, black tips indicate DyP-type Peroxidase family while the blue tips represent Catalase-peroxidases. The blue triangular tip indicates the protein sequence obtained in this study (EadPrx). The UniProtKB reference numbers/GenBank accession number* of the proteins are indicated in parentheses.

El: *Erythrobacter litoralis*, Msp: *Mesorhizobium* sp., Nw: *Nitrobacter winogradskyi*, Pde: *Paracoccus denitrificans*, Ret: *Rhizobium etli*, RHsp: *Rhizobium* sp., Mlo: *Mesorhizobium loti*, Rp: *Rhodopseudomonas palustris*, Rru: *Rhodospirillum rubrum*, Go: *Gluconobacter oxydans*, Eco: *Escherichia coli*, Sbo: *Shigella boydii*, Sso: *Shigella sonnei*, Sf: *Shigella flexneri*, Oant: *Ochrobactrum anthropic* EadPrx: *Ensifer adhaerens* NWODO-2 Peroxidase, DyPPrx: Dye-decolourizing Peroxidase, KatG: Catalase-peroxidase.

6.4. Discussion

The utility of peroxidase in different industrial sectors has led to an increased demand which, probably cannot be met by horseradish peroxidase (HRP), the major commercially available peroxidase. Hence, the imperativeness of novel bacteria with improved peroxidase production capacity. This study improved extracellular peroxidase production by *E. adhaerens* NWODO-2 by optimizing the process parameters and amending the composition of the production medium.

Bacteria growth has been linked to the constant secretion of extracellular enzymes into the culture medium (McCarthy, 1987; Niladevi and Prema, 2008; Musengi *et al.*, 2014). Therefore, factors capable of influencing bacteria growth such as pH, temperature and agitation as well as the composition of the medium will invariably play a significant role in enzyme production by the bacteria.

The pH of the cultivation environment has a significant influence on the electric charge of the microbial cell, consequently affecting the absorption of nutrient and intracellular enzymatic activities (Salehizadeh and Shojaosadati, 2001; Makapela *et al.*, 2016). The test strain, *E. adhaerens* NWODO-2 produced peroxidase within a pH range of 5.0 - 9.0 with the optimum production observed at an initial medium pH 7.0 (Fig. 6.1). This finding is in accordance with the works of Rob *et al.* (1997) and Nour El-Dein *et al.* (2014), in which optimum peroxidase production by *Streptomyces avermitilis* UAH30 and *Streptomyces* sp. K37 was reported at pH 7 and 7.5, respectively. However, Musengi *et al.* (2014) observed optimum peroxidase production by *Streptomyces* sp. BSII#1 at pH 8. It is worthy of note that the ability of the test organism to produce peroxidase optimally at a neutral pH augurs well for biotechnological applications as the large volume of acid and base required for pH adjustment would have been saved (Xia *et al.*, 2008). Consequently, reducing the cost of peroxidase production.

The optimal peroxidase production by *E. adhaerens* NWODO-2 occurred at 30 ^{o}C (Fig. 6.2). Although, no comparative data was found on optimal temperature for peroxidase production by Ensifer species, our finding agrees with the result obtained by Rajkumar *et al.* (2013) where optimum peroxidase production by a *Bacillus* sp. was also observed at 30 ^{o}C. Nevertheless, Rao and Kavya (2014) and Musengi *et al.* (2014) reported 37 ^{o}C as the optimal temperature for peroxidase production by *Bacillus subtilis* and *Streptomyces* sp. BSII#1 respectively whereas Nour El-Dein *et al.* (2014) reported 40 ^{o}C as the optimal temperature for peroxidase production by *Streptomyces* sp. K37. The reason for the discrepancies in the optimal temperature for peroxidase production by the different bacteria species is unclear. However, all the optimal temperatures fall within the mesophilic range.

The marked decrease in peroxidase production by *E. adhaerens* NWODO-2 at temperatures below and above 30 ^{o}C (Fig. 6.2) may probably, be due to reduction in metabolic activities which may consequently inhibit the organism growth and enzyme biosynthesis (Ray *et al.,* 2007).

Agitation tends to affect the level of aeration and proper mixing of nutrients in the production medium, thereby making nutrient more accessible to the organism (Giavasis *et al.,* 2006). Peroxidase production by *E. adhaerens* NWODO-2 was affected by agitation as there was a slight increase in peroxidase production at various agitation speeds investigated (50 – 200 rpm) when compared with static condition (Fig. 6.3). This finding is consistent with previous studies where agitation affected the level of production of enzymes (Sepahy *et al.,* 2011; Patil, 2014; Fatokun *et al.,* 2016). In this study, the agitation rate of 100 rpm was most favourable for peroxidase production by the test organism but Patil (2014) reported 180 rpm as the optimal agitation rate for lignin peroxidase production by *Bacillus megaterium*. This therefore suggests that different organisms have different favourable agitation rates during fermentation.

Although, there is dearth of information on the induction of peroxidases by lignin monomers in bacteria, a number of studies have reported the inductive effect of lignin monomers on peroxidase production by ligninolytic organisms, predominantly, fungi (Niku-Paavola *et al.,* 1990; Mester *et al.,* 1995; Couto *et al.,* 1999). In this study, the effects of guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid on peroxidase production were assessed. Our findings showed that guaiacol induced peroxidase production by *E. adhaerens* NWODO-2 as it increased the enzyme production by about 50 % (Fig.6.4). On the other hand, veratryl alcohol and ferulic acid did not

have an inductive effect on peroxidase production by the test organism while vanillin and vanillic acid seemed to promote repression of the enzyme synthesis. This finding is contrary to the result obtained by Musengi *et al.* (2014) where veratryl alcohol was the best inducer of peroxidase production by *Streptomyces* sp. strain BSII#1 but there is no comparative data on peroxidase production by Ensifer species. Hence, inducers may be species-specific.

The effects of nature and concentration of nitrogen sources on ligninolytic enzyme production have been extensively studied, especially in fungi (Kachlishvili *et al.,* 2005; Mikiashvili *et al.,* 2006; Stajic *et al.,* 2006) but the findings are not always consistent (Niladevi and Prema, 2008). Fermentation media with sufficient nitrogen has enhanced ligninolytic enzyme production in some fungi (Kaal *et al.,* 1995). More so, nitrogen-limited conditions have enhanced production of ligninolytic enzymes (Mester and Field, 1997; Gianfreda *et al.,* 1999; Galhaup *et al.,* 2002) while in some cases, production of lignin modifying enzymes has been limited by high nitrogen concentration (Buswell, 1992). In this study, supplementation of the fermentation medium with inorganic nitrogen sources enhanced peroxidase production by *E. adhaerens* NWODO-2, with ammonium sulphate [$(NH_4)_2SO_4$] giving the optimum peroxidase yield (Fig. 6.5). This finding contradicts the result obtained by Kachlishvili *et al.* (2005) where manganese peroxidase production by some white-rot basidiomycetes was repressed by additional nitrogen. This was also corroborated by Mikiashvili *et al.* (2006) who reported that supplementation of defined fermentation medium with inorganic nitrogen sources decreased the production of lignin modifying enzymes including peroxidase by *Pleurotus ostreatus*.

The production of peroxidase by *E. adhaerens* NWODO-2 is growth-associated as the enzyme increased significantly at the logarithmic growth phase with maximum productivity attained at 48 h (Fig. 6.6). The sharp decline in peroxidase production observed at 72 h, might probably be attributed to nutrient depletion or proteolytic activities (Papagianni and Moo-Young, 2002; Fatokun *et al.,* 2016). This finding is contradictory to previous related reports where optimum peroxidase production by other bacteria species was attained at 72 h of incubation (Rob *et al.,* 1997; Tuncer *et al.,* 1999; Nour El-Dein *et al.,* 2014). The detection of an extracellular enzyme activity in the absence of hydrogen peroxide, the typical peroxidase activator is noteworthy, as this may suggest probable laccase production by *E. adhaerens* NWODO-2.

High cost of production is a major challenge to the industrial application potentials of peroxidases. Hence, the imperativeness of cost effective means of peroxidase production. The use of cheap substrates as alternative carbon sources for fermentation has been suggested as an effective way of reducing the cost of enzyme production (Falade *et al.,* 2017a). The abundance, availability and renewable nature of lignocellulosic materials confer on them the perfect candidature of cheap carbon sources. Agricultural residues such as sawdust, wheat straw, corn cobs, rice straw, peapods etc have been valorized for production of various ligninocellulolytic enzymes (Knezevic *et al.,* 2013; Saratale *et al.,* 2014; Sharma *et al.,* 2015; Neifar *et al.,* 2016; Olajuyigbe and Ogunyewo, 2016) through solid state and submerged fermentation processes.

SSF is perhaps, more promising for optimum valorization of agricultural residues for enzyme production as enzyme production by bacteria under solid state fermentation is reported to be economical (Muthukumarasamy and Murugan, 2014). SSF is also characterized by high production yield, low wastewater output and decrease in energy demand (Niladevi *et al.,* 2007). This study valorized selected agricultural residues (sawdust, wheat straw and corn stover) for peroxidase production under SSF. *E. adhaerens* NWODO-2 gave the highest exoperoxidase yield when grown on sawdust as solid substrate (Table 6.1). This finding is consistent with previous related studies (Knezevic *et al.,* 2013: Kamsani *et al.,* 2016), where high yield of peroxidase was induced by sawdust as solid substrate under SSF. This finding could be attributed to the inductive effect of the phenolic and non-phenolic components of sawdust. However, there was no enzyme activity detected in the absence of hydrogen peroxide when grown on sawdust and wheat straw, suggesting that the substrates may perhaps repress laccase production by *E adhaerens* NWODO-2. Nonetheless, corn stover induced extracellular enzyme activity in the absence of hydrogen peroxide, indicating likely laccase production. This suggests that corn stover might possess phenolic compounds with inductive laccase effect.

Molecular optimization and genetic engineering seem to be the best option for increased enzyme production. Hence, the imperativeness of detecting the gene responsible for the expression of the enzyme of interest in an organism. Phylogenetic analysis of the protein expressed by the amplified gene (Fig. 6.8) suggests it's a catalase-peroxidase. Bacterial catalase-peroxidases (KatG) belong to class I peroxidases of peroxidase-catalase superfamily of heme-peroxidases (Zamocky *et al.,*

2008). Other members of class I peroxidases include yeast cytochrome c peroxidases and ascorbate peroxidases.

KatG gene encodes the expression of both catalase and peroxidase activity (Zamocky and Obinger, 2010) in an organism. This therefore suggests that peroxidase activity expressed in *Ensifer adhaerens* NWODO-2 may be attributed to the presence of KatG gene in the new bacteria strain. Hence, the gene could be explored for large scale production of catalase and peroxidase through genetic engineering, which has proven difficult in fungi (Bugg *et al.,* 2011). Besides, the presence of KatG gene in *Ensifer adhaerens* NWODO-2 also attests to its ligninolytic potential as bacteria catalase-peroxidase has recently been implicated in degradation of lignocellulose (Brown *et al.,* 2011; de Gonzalo *et al.,* 2016).

6.5. Conclusion

Peroxidase production by *E. adhaerens* NWODO-2 was optimal at a neutral pH, mesophilic temperature, mild agitation rate and relatively short incubation time. In this study, peroxidase production increased by over 100% under optimum conditions with guaiacol as an inducer and sawdust as the best substrate for solid state fermentation. The ability of the new strain to utilize agricultural residues as cheap renewable substrates for peroxidase production serve as a cost-effective means of enzyme production which could be employed for large scale production and consequent biotechnological applications. Furthermore, a novel catalase-peroxidase encoding gene detected in *E. adhaerens* NWODO-2 may be responsible for the peroxidase activity expressed in the organism.

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CHAPTER SEVEN

Peroxidase production by a novel ligninolytic proteobacteria strain: *Raoultella ornithinolytica* OKOH-1

(*Submitted to AMB Express*)

Abstract

Aim: To enhance peroxidase production by *Raoultella ornithinolytica* OKOH-1 (KX640917).

Methods and Results: Peroxidase production by *Raoultella ornithinolytica* OKOH-1 was enhanced by determining the optimum culture conditions (initial pH, incubation temperature and agitation speed) using the classical process of one variable at a time. The carbon and nitrogen sources (kraft lignin and yeast extract) were supplemented with some lignin model compounds and inorganic nitrogen compounds, respectively. Subsequently, the time-course assay was carried out under optimized conditions. Then, some lignocellulosic wastes were valorized for peroxidase production under solid state fermentation. Peroxidase production was optimal at initial pH 5, incubation temperature of 35 ^{o}C and agitation speed of 150 rpm with guaiacol and ammonium chloride as the best inducer and nitrogen supplement, respectively. Peroxidase production by *Raoultella ornithinolytica* strain OKOH-1 was optimal at 72 h with 15.17 ± 0.82 U mL^{-1} and specific productivity of 16.48 ± 0.89 U mg^{-1}. A simultaneous production of a non-peroxide dependent extracellular enzyme (12.54 ± 0.41 U mL^{-1}) which suggests probable laccase production was observed with specific productivity of 13.63±0.45 U mg^{-1}. Furthermore, sawdust gave the best peroxidase yield (15.21 ± 2.48 U mg^{-1}).

Conclusions: Peroxidase production by *Raoultella ornithinolytica* strain OKOH-1 increased by 3.40-fold under optimum conditions with guaiacol as the most effective inducer and sawdust as the best solid substrate for solid state fermentation.

Significance and Impact of the Study: Over the years, actinomycetes have been the major known peroxidase-producing bacteria. It is therefore imperative to explore other classes of bacteria with untapped peroxidase production potentials that can be exploited for various biotechnological applications.

Keywords: Enzyme production, lignocellulosic wastes, microbial peroxidase, proteobacteria, valorization.

7.1. Introduction

Peroxidases (EC 1.11.1) are a group of oxidoreductive enzymes that oxidize various organic and inorganic substrates with hydrogen peroxide as an electron acceptor (Falade *et al.,* 2017a). The large presence of peroxidases in plants, animals and microbes where they perform different physiological functions has been reported (Battistuzzi *et al.,* 2010). Some of the physiological functions of peroxidases include involvement in innate immune system (Söderhall, 1999), protection against toxic peroxide (Dunford, 1999), peroxide sensing, protection against oxidative stress, cell wall biosynthesis and oxidation of poisonous compounds (Smulevich *et al.,* 2006; Battistuzzi *et al.,* 2010; Martins *et al.,* 2013).

Besides the physiological functions of peroxidases, their potential for biotechnological applications span through various industrial sectors including energy, textile, bioremediation, cosmeceutical and dermatological industries (Falade *et al.,* 2017a). Peroxidases have been applied in the development of biosensors (Jia *et al.,* 2002) and analysis/diagnostic kits (Agostini *et al.,* 2002). They have also been implicated in lignin degradation (Hatakka *et al.,* 2003); a function specific to class II peroxidase-catalase superfamily of heme-peroxidases including lignin peroxidase, manganese peroxidase and versatile peroxidase. Their potentials for development of skin-lightening agents and removal of endocrine distrupting chemicals (EDCs) in wastewater have recently been reported (Taboada-Puig *et al.,* 2015; Draelos, 2015). Other biotechnological applications of peroxidases include but not limited to biopulping and biobleaching (Hatakka *et al.,* 2003), oxidation of several organic substrates including phenolic and non-phenolic compounds (Cheng *et al.,* 2006) and degradation of xenobiotics such as synthetic dyes (Kalyani *et al.,* 2011).

Given the high-utility potential of peroxidases, their production in large amount is of utmost importance as enhanced enzyme production is one of the significant requirements for an effective bio-catalytic process. White rot fungi have been identified as the best producers of oxidative enzymes including peroxidases. More so, several efforts have been made to optimize the production of peroxidases by fungi (Ikehata *et al.,* 2004; Urek and Pazarlioglu, 2007; Irshad and Asgher, 2011; Hariharan and Nambisan, 2013). Nonetheless, bacteria seem to present a striking advantage over fungi as peroxidase producer as they can easily be cultured in defined media and peroxidase production optimized accordingly. Moreover, production of extracellular peroxidases by bacteria has been reported (Mercer *et al.,* 1996; Tuncer *et al.,* 1999, 2004, 2009; Dawkar *et al.,*

2009; Kalyani *et al.,* 2011; Musengi *et al.,* 2014). However, most of the reported peroxidase-secreting bacteria are actinomycetes, predominantly Streptomyces species. It is worthy of note that over-dependent on actinomycetes for enzyme production has led to a neglect of other classes of bacteria with perhaps, better potential for enzyme production. Hence, the exploration of other classes of bacteria for enhanced extracellular peroxidase production is imperative.

The emerging ligninolytic activity of proteobacteria (Bugg *et al.,* 2011; Taylor *et al.,* 2012) confers on them the perfect candidature for production of ligninolytic enzymes including peroxidases and laccase. Production of peroxidase and laccase by Pseudomonas species, a gamma proteobacteria has been reported (Kalyani *et al.,* 2011; Neifar *et al.,* 2016). It is therefore evident that proteobacteria is a reservoir of unexploited peroxidase production potential that can be explored for various industrial applications. Our preliminary study identified *Raoultella ornithinolytica* OKOH-1 as a ligninolytic bacteria with a good potential for peroxidase production (Falade *et al.,* 2017b). This study therefore aims at enhancing peroxidase production by *R. ornithinolytica* OKOH-1 using optimized culture conditions.

7.2. Materials and methods

7.2.1. Isolation, screening and molecular identification

The organism was isolated from sediments of Tyhume River in Alice, South Africa by enrichment technique using kraft lignin (Sigma-Aldrich, South Africa) as the sole carbon source (Sasikumar *et al.,* 2014). Subsequently, the isolate was carefully chosen on the basis of its ligninolytic and peroxidase production potential (Falade *et al.,* 2017b). The 16S rDNA partial sequence analysis showed that the bacterial strain had 99% similarity to *R. ornithinolytica* strain G.W-CD.10 (Falade *et al.,* 2017b). Its nucleotide sequence is available in the GenBank of the National Centre for Biotechnology Information (NCBI) as *Raoultella ornithinolytica* strain OKOH-1 under the accession number, KX640917.

7.2.2. Peroxidase production and extraction of crude enzyme

Peroxidase was produced using the method of Falade *et al.* (2017b). The culture was subsequently incubated at 30 ^{o}C and 140 rpm for 48 h, a period over which the organism showed peroxidase activity during the initial screening. Thereafter, cultures were aseptically withdrawn and the crude

enzyme extracted as described by Falade *et al.* (2017b). The recovered supernatant was then utilized as crude enzyme for peroxidase assay.

7.2.3. Peroxidase assay

The peroxidase activity was evaluated by the rate of hydrogen peroxide-dependent oxidation of pyrogallol to purpurogallin using the modified method of Chance and Maehly (1955) described by Falade *et al.* (2017b).

7.2.4. Determination of optimal parameters for peroxidase production

The optimal parameters for peroxidase production were determined as described by Fatokun *et al.* (2016). Briefly, the various culture parameters were adjusted for optimum peroxidase production using the conventional method of one factor at a time. Then the determined optimized conditions were used in subsequent fermentation (Fatokun *et al.*, 2016).

7.2.4.1. Determination of optimal initial pH

The optimal initial pH for peroxidase production was determined by growing *R. ornithinolytica* OKOH-1 in a kraft lignin modified fermentation medium with pH ranging from 3 to 11 at 30 ^{o}C.

7.2.4.2. Determination of optimal incubation temperature

The optimal incubation temperature for peroxidase production was determined by growing *R. ornithinolytica* OKOH-1 for 48 h at a varying temperature range of 20 to 45 ^{o}C at 5 ^{o}C intervals in a kraft lignin modified fermentation medium of pH 5, being the optimal initial pH for peroxidase production.

7.2.4.3. Determination of optimal agitation speed

To determine the optimal agitation speed for peroxidase production by *R. ornithinolytica* OKOH-1, the strain was grown for 48 h in a kraft lignin modified fermentation medium at the optimal temperature (35 ^{o}C) and initial pH (pH 5) for peroxidase production at varying agitation speeds ranging from static condition to 200 rpm at 50 rpm intervals.

7.2.4.4. Effect of carbon supplementations on peroxidase production

The effect of carbon supplementations on peroxidase production was assessed by growing *R. ornithinolytica* OKOH-1 in a kraft lignin modified fermentation medium supplemented with 1 mmol L^{-1} of different lignin model compounds: guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid (Musengi *et al.*, 2014) at the optimal temperature (35 ^{o}C), initial pH (pH 5) and agitation speed (150 rpm) for peroxidase production.

7.2.4.5. Effect of nitrogen supplementations on peroxidase production

The effect of nitrogen supplementations on peroxidase production was assessed by growing *R. ornithinolytica* OKOH-1 in a kraft lignin modified fermentation medium where yeast extract was supplemented with different inorganic nitrogen sources (ammonium nitrate, ammonium chloride and ammonium sulphate) at the optimal temperature (35 ^{o}C), initial pH (pH 5) and agitation speed (150 rpm) for peroxidase production, with guaiacol (1 mmol L^{-1}) being used as an inducer.

7.2.5. Kinetics of peroxidase production and bacterial growth

The peroxidase production by *R. ornithinolytica* OKOH-1 as well as its growth kinetics were assessed as described by Tuncer *et al.* (1999) with slight modifications. In brief, the proteobacteria strain was grown in kraft lignin modified fermentation medium under optimized conditions for 144 h. Subsequently, the culture was intermittently withdrawn at 24 h interval (Tuncer *et al.*, 1999) and assayed for peroxidase and non-peroxide dependent enzyme production, as well as total protein concentration. Also, the cell growth was monitored by measuring the optical density (OD) of the culture at 600 nm.

7.2.6. Protein estimation

The total protein was estimated by using the Bradford method (Bradford, 1976). Briefly, 250 µL of Bradford reagent was added to 10 µL of the supernatant in a 96-well microtitre plate while 10 µL of distilled water was used in place of the sample in the blank. The mixture was subsequently incubated at room temperature for 15 min. Thereafter the absorbance was measured at 595 nm using SynergyMx 96-well microtitre plate reader (BioTeK Instruments). The protein concentration was extrapolated from the standard curve constructed using bovine serum albumin (BSA). The specific enzyme productivity was expressed as U/mg protein.

7.2.7. Valorization of lignocellulosic wastes for peroxidase production under solid state fermentation (SSF)

Selected lignocellulosic wastes including sawdust, wheat straw and corn stover were valorized for peroxidase production under SSF. The sawdust was obtained from a sawmill in Alice metropolis, South Africa while the wheat straw and corn stover were obtained from the agricultural research farm of the University of Fort Hare in Alice, Eastern Cape, South Africa. Thereafter, the lignocellulosic materials were washed, air-dried and ground with a laboratory milling machine (Lasec, South Africa). The milled products were thereafter sieved into powder form (with size less than 500 µm). The processed lignocellulosic materials were subsequently used as solid substrate for peroxidase production by *R. ornithinolytica* OKOH-1 using the modified method of Neifar *et al.* (2016). Briefly, 5g of substrate was weighed into 100 mL conical flasks and dampened with 10 - 20 mL of minimal salt medium (4.55 g L^{-1} K_2HPO_4, 0.53 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 5 g L^{-1} NH_4Cl, 0.1 g L^{-1} yeast extract) supplemented with 1 mmol L^{-1} of guaiacol, with an initial pH of 5, being the optimal initial pH for peroxidase production by the strain under investigation. Then the preparations in the flasks were autoclaved at 121 ^oC for 15 min and thereafter inoculated with 1 mL of the bacterial suspension in normal saline (O.D. 600 nm ≈ 1.0). After 72 h incubation (the time for optimal peroxidase production by *R. ornithinolytica* OKOH-1), 20 mL of 100 mmol L^{-1} potassium phosphate buffer (pH 6) was added to the flasks and stirred for 30 min for the extraction of crude enzyme. Subsequently, the contents of the flasks were centrifuged at 15000 rpm for 10 min at 4 ^oC using benchtop cold centrifuge (SIGMA 1-14K), and the recovered supernatant was used as crude enzyme for peroxidase assay.

7.2.8. Data analysis

Results of replicates were expressed as mean ± standard deviation (STD) using Microsoft Excel Spreadsheet. Data were subsequently subjected to one-way ANOVA using GraphPad Prism 7 followed by Tukey's Multiple Comparison Test. Significance was accepted at $P \leq 0.05$.

7.3. Results

7.3.1. Optimal parameters

7.3.1.1. Optimal initial pH for peroxidase production

The optimal initial medium pH was determined and the results are presented in Fig. 7.1. The results showed that *R. ornithinolytica* OKOH-1 produced peroxidase over a wide pH range of 5 to 11 with the optimum production observed at pH 5 (3.44 ± 0.64 U mL^{-1}). However, no peroxidase activity was detected at pH 3 and 4. Although there exists a significant difference ($P < 0.05$) in peroxidase production at pH 5 compared to other pH values, there was no significant difference ($P > 0.05$) in peroxidase production at pH 6 (1.52 ± 0.00 U mL^{-1}), pH 7 (1.93 ± 0.41 U mL^{-1}), pH 8 (1.63 ± 0.00 U mL^{-1}) and pH 9 (1.23 ± 0.06 U mL^{-1}).

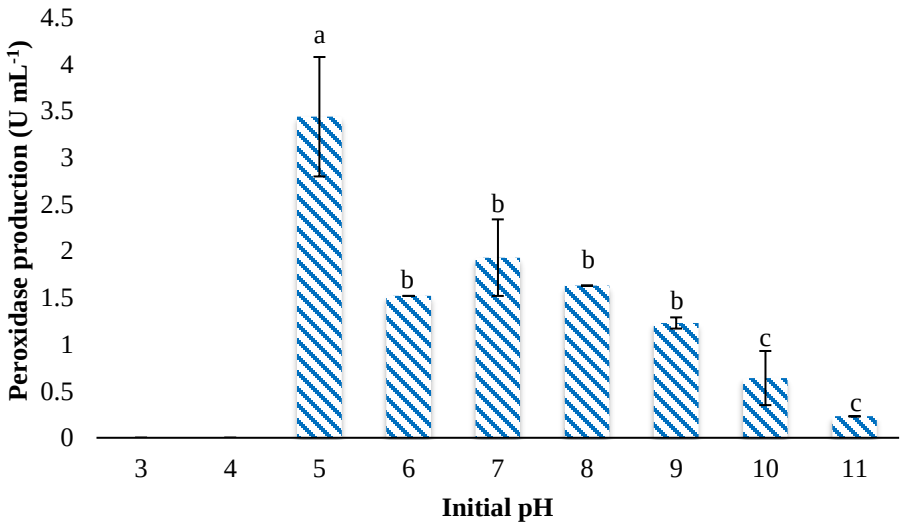

Fig. 7.1. Determination of optimal initial pH for peroxidase production by *R. ornithinolytica* OKOH-1 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different ($P > 0.05$).

7.3.1.2. Optimal incubation temperature for peroxidase production

The result on the determination of optimal incubation temperature for peroxidase production by *R. ornithinolytica* OKOH-1 is presented in Fig. 7.2. The results showed a significant difference ($P < 0.05$) in peroxidase production across the temperatures: 20 – 45 ^{o}C with optimal peroxidase production observed at an incubation temperature of 35 ^{o}C (5.25 ± 0.00 U mL^{-1}). Nevertheless, Tukey's multiple comparison test revealed no significant difference ($P > 0.05$) in the peroxidase production at 20 ^{o}C (1.70 ± 0.87 U mL^{-1}), 25 ^{o}C (1.87 ± 0.70 U mL^{-1}), 40 ^{o}C (1.11 ± 0.17 U mL^{-1}) and 45 ^{o}C (1.81 ± 0.29 U mL^{-1}).

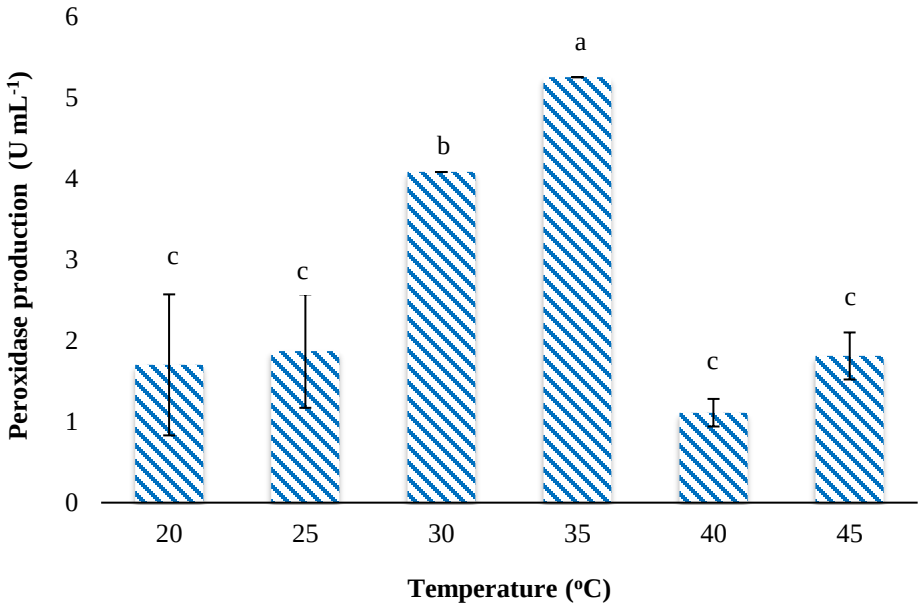

Fig. 7.2. Determination of optimal incubation temperature for peroxidase production by *R. ornithinolytica* OKOH-1 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different ($P > 0.05$).

7.3.1.3. Optimal agitation speed for peroxidase production

Fig. 7.3 shows the results on the determination of optimal agitation speed for peroxidase production by *R. ornithinolytica* strain OKOH-1. The results revealed that there was a significant difference ($P < 0.05$) in peroxidase production by *R. ornithinolytica* strain OKOH-1 in static condition, and across all agitation speeds (50-200 rpm) with the optimal peroxidase production observed at agitation speed of 150 rpm (9.45 ± 2.57 U mL^{-1}). However, Tukey's multiple comparison test revealed no significant difference ($P > 0.05$) in the peroxidase production at 50 rpm (2.33 ± 0.00 U mL^{-1}), 100 rpm (2.33 ± 0.35 U mL^{-1}) and 200 rpm (2.10 ± 0.00 U mL^{-1}).

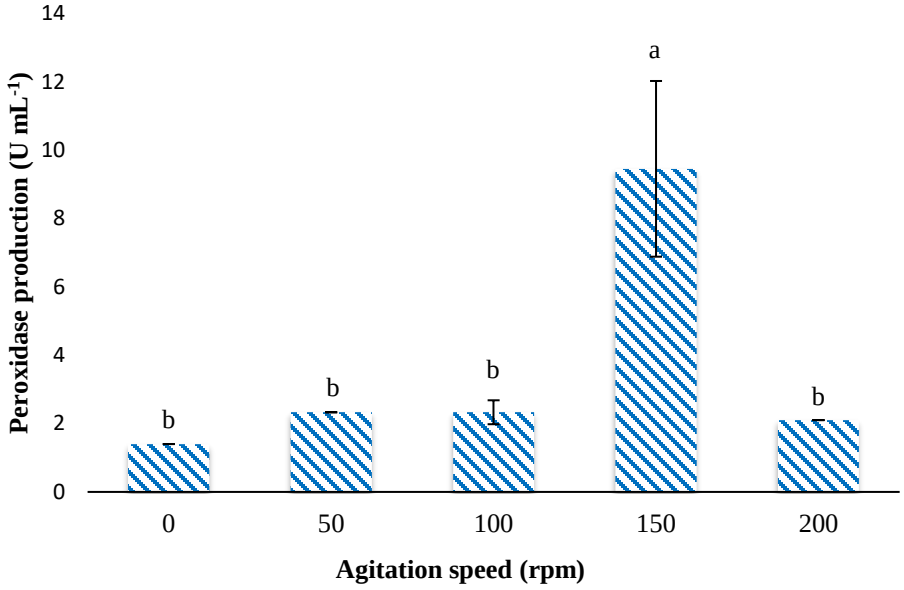

Fig. 7.3. Determination of optimal agitation speed for peroxidase production by *R. ornithinolytica* OKOH-1 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different ($P > 0.05$).

7.3.1.4. Effect of carbon supplementation on peroxidase production

The effects of supplementing the kraft lignin (KL) modified fermentation medium with 1 mmol L^{-1} of various lignin model compounds (Guaiacol-GA, Veratryl alcohol-VALC, vanillin-VAN, vanillic acid-VA and ferullic acid-FA) are shown in Fig. 7.4. The result revealed a significant

difference ($P < 0.05$) in peroxidase production by *R. ornithinolytica* strain OKOH-1 grown in kraft lignin modified production medium supplemented with lignin model compounds (KL+GA; KL+VALC; KL+VAN; KL+VA; and KL+FA) when compared with non-supplemented production medium (KL), which served as the control. All the lignin model compounds induced peroxidase production by *R. ornithinolytica* strain OKOH-1 with the highest inducing effect produced by guaiacol (7.82 ± 0.00 U mL^{-1}).

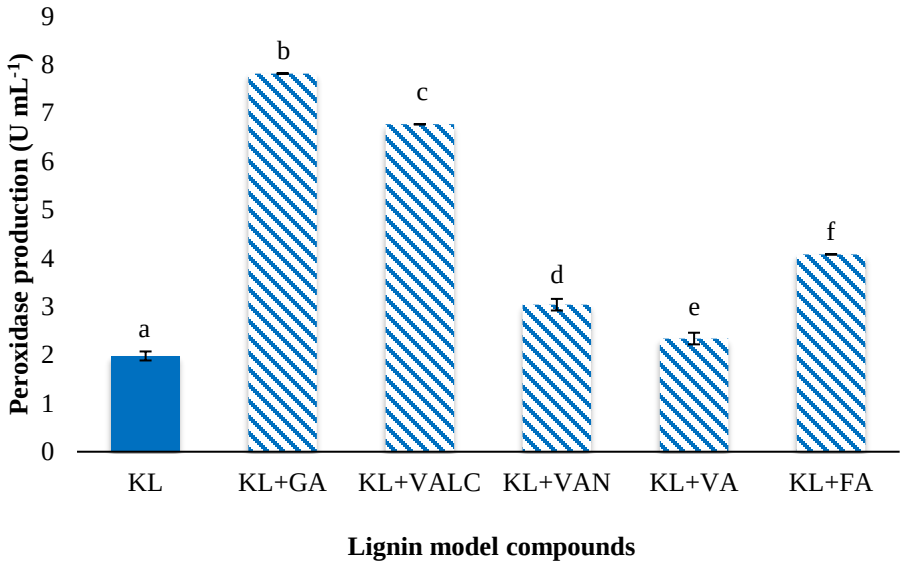

Fig. 7.4. Effect of carbon supplementations on peroxidase production by *R. ornithinolytica* OKOH-1 (Each column represent mean ± standard deviation; n=3). Error bars with different alphabet are significantly different ($P< 0.05$). KL: kraft lignin (control), GA: guaiacol, VALC: veratryl alcohol, VAN: vanillin, VA: vanillic acid, FA: ferullic acid.

7.3.1.5. Effect of nitrogen supplementations on peroxidase production

The effect of supplementing yeast extract with different inorganic nitrogen sources is presented in Fig. 7.5. The results showed that there was no significant difference ($P > 0.05$) in peroxidase production by *R. ornithinolytica* OKOH-1 grown in the production medium supplemented with inorganic nitrogen sources (Ammonium Nitrate; Ammonium Chloride; and Ammonium Sulphate). However, the supplementation of the organic nitrogen source in the production medium, yeast

extract, with ammonium chloride (YE + Ammonium Chloride) yielded the maximum peroxidase production (10.09 ± 1.34 U mL^{-1}).

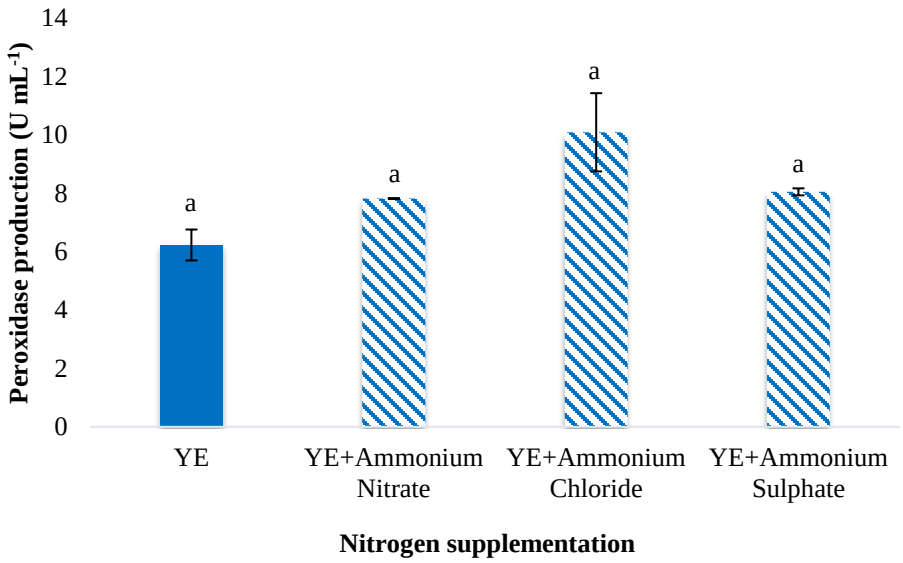

Fig. 7.5. Effect of nitrogen supplementations on peroxidase production by *R. ornithinolytica* OKOH-1 (Each column represent mean ± standard deviation; n=3). Error bars with the same alphabet are not significantly different (*P> 0.05*). YE: Yeast Extract.

7.3.2. Kinetics of peroxidase production and bacterial growth

The Raoultella species was assessed for peroxidase and non-peroxide dependent extracellular enzyme production over an incubation period of 144 h and the results are presented in Fig. 7.6. The results indicated that *R. ornithinolytica* strain OKOH-1 attained optimum peroxidase production (15.16 ± 0.82 U mL^{-1}) and non-peroxide dependent extracellular enzyme production (12.54 ± 0.41 U mL^{-1}) at 72 h (Fig. 7.6a) with specific productivity of 16.48 ± 0.89 U mg^{-1} protein and 13.63 ± 0.45 U mg^{-1} protein respectively (Fig. 7.6b), corresponding to the early stationary growth phase. However, there was a sharp decrease in peroxidase production (0.47 ± 0.00 U mL^{-1}) and non-peroxide dependent extracellular enzyme production (2.68 ± 0.00 U mL^{-1}) at 96 h with

specific productivity of 0.52 ± 0.00 U mg^{-1} protein and 2.95 ± 0.00 U mg^{-1} protein, respectively (Fig. 7.6).

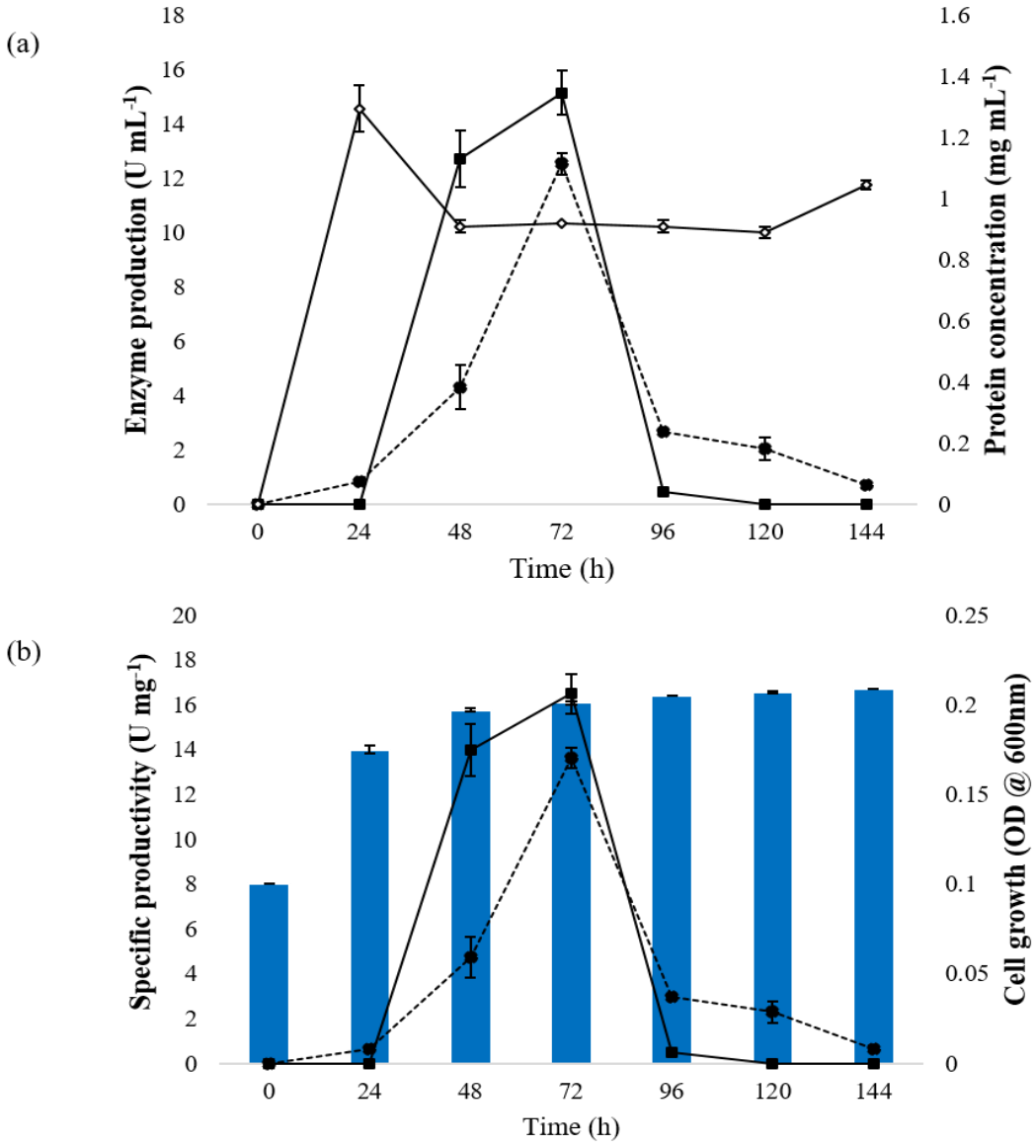

Fig. 7. 6. Kinetics of extracellular enzyme production by *R. ornithinolytica* OKOH-1. (a). Enzyme production: peroxidase (—■—) and non-peroxide dependent enzyme (---✹---); and protein concentration (—◇—). (b). Specific productivity: peroxidase (—■—) and non-peroxide dependent enzyme (---✹---); and cell growth (▬). Each column represent mean ± standard deviation (n=3).

7.3.3. Valorization of lignocellulosic wastes for peroxidase production

The results of valorization of lignocellulosic wastes for peroxidase production by *R. ornithinolytica* strain OKOH-1 under SSF are presented in Table 7.1. The results showed that there was a significant difference ($P < 0.05$) in peroxidase production by the test bacteria when grown on all the selected substrates with sawdust having the best yield (15.21 ± 2.48 U mg^{-1}) while the lowest yield was observed on corn stover (1.30 ± 0.00 U mg^{-1}). However, there was no significant difference ($P > 0.05$) in non-peroxide dependent enzyme produced by *R. ornithinolytica* strain OKOH-1 grown on all the substrates. Nevertheless, sawdust also gave the highest yield (6.73 ± 1.76 U mg^{-1}) while corn stover had the lowest yield (3.78 ± 0.14 U mg^{-1}).

Table 7.1. Valorization of lignocellulosic wastes for peroxidase production under SSF

Lignocellulosic wastes	**Protein concentration (mg mL^{-1})**	**Peroxidase**		**Non-peroxide dependent enzyme**	
		Enzyme production (U mL^{-1})	Specific productivity (U mg^{-1})	Enzyme production (U mL^{-1})	Specific productivity (U mg^{-1})
Sawdust	0.165 ± 0.00^{a}	2.51 ± 0.41^{a}	15.21 ± 2.48^{a}	1.11 ± 0.29^{a}	6.73 ± 1.76^{a}
Wheat straw	0.927 ± 0.048^{b}	6.65 ± 0.47^{b}	7.18 ± 0.51^{b}	4.73 ± 0.65^{b}	5.09 ± 0.69^{a}
Corn stover	1.250 ± 0.00^{b}	1.63 ± 0.00^{a}	1.30 ± 0.00^{c}	4.73 ± 0.18^{b}	3.78 ± 0.14^{a}

Values represent mean ± standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different ($P > 0.05$). SSF: solid state fermentation.

7.4. Discussion

Given the high-utility potential of peroxidases, the search for novel bacteria with enhanced peroxidase production is imperative. In this study, we enhanced peroxidase production by *R. ornithinolytica* OKOH-1, a novel ligninolytic proteobacterial strain by optimizing the different culture conditions and manipulating the fermentation medium compositions.

The continuous secretion of ligninolytic enzymes including peroxidase into the fermentation medium has been associated with bacterial growth (McCarthy, 1987; Niladevi and Prema, 2008; Musengi *et al.*, 2014). Consequently, factors affecting the growth of bacteria including initial pH

of the medium, incubation temperature and agitation speed as well as the medium compositions will have influence on peroxidase production by *R. ornithinolytica* OKOH-1.

R. ornithinolytica OKOH-1 was able to grow within a wide pH range (5.0 - 11.0) with maximum peroxidase production at slightly acidic initial medium pH of 5.0 (Fig. 7.1), a characteristic that indicates the ability of the bacteria to tolerate an acidic environment which also augurs well for its industrial relevance. This finding agrees with the previous study by Mmango-Kaseke *et al.* (2016) who reported optimal cellulase production by *Micrococcus luteus* strain SAMRC-UFH3 at pH 5.

The bacterial strain under investigation grew within the mesophilic temperature range (20 – 45 ^{o}C) with maximum peroxidase production at 35 ^{o}C (Fig. 7.2), which is in accordance with the findings of the previous study by Tuncer *et al.* (2004) who reported optimal production of endoxylanase, endoglucanase and peroxidase by *Streptomyces* sp. F2621 at 35 ^{o}C. This is also corroborated by Fatokun *et al.* (2016) who reported optimal xylanase production by *Streptomyces albidoflavus* strain SAMRC-UFH 5 at 35 ^{o}C. However, Nour El-Dein *et al.* (2014) reported 40 ^{o}C as the optimal temperature for peroxidase production by *Streptomyces* sp. K37 while Rao and Kavya (2014) reported 37 ^{o}C as optimal temperature for peroxidase production by *Bacillus subtilis.* Although there were discrepancies in the reported optimal temperatures for the production of lignocellulolytic enzymes which might perhaps be due to the environment from which the organisms were isolated, all the temperatures reported fall within the mesophilic range. The significant decrease in peroxidase production by *R. ornithinolytica* OKOH-1 observed at temperatures below and above 35 ^{o}C (Fig. 7.2) might be attributed to the reduction in metabolic activities which may lead to inhibition of the bacterial growth and enzyme synthesis (Ray *et al.* 2007).

Moreover, peroxidase production by *R. ornithinolytica* OKOH-1 was affected by agitation with maximum peroxidase production observed at 150 rpm (Fig. 7.3), which is consistent with the findings of Fatokun *et al.* (2016) on xylanase production by *Streptomyces albidoflavus* strain SAMRC-UFH 5 but contrary to that of Sepahy *et al.* (2011) who reported maximum xylanase production by *Bacillus mojavensis* AG137 at 200 rpm. Also, Patil (2014) reported optimal lignin peroxidase production by *Bacillus megaterium* at 180 rpm.

In this study, the agitation rate of 150 rpm was more favourable for peroxidase production, a finding which is in agreement with the observation by Giavasis *et al.* (2006) that agitation tends to

affect the level of aeration and proper mixing of nutrients in the production medium, thereby making nutrient more accessible to the organism.

The induction of peroxidases in ligninolytic organisms by lignin model compounds has been reported from a number of studies (Niku-Paavola *et al.,* 1990; Mester *et al.,* 1995; Couto *et al.,* 1999). Although, there is dearth of information on the inductive effect of lignin model compounds on peroxidase production by bacteria, there is quite a number of reports on the influence of lignin model compounds on laccase production (Dekker *et al.,* 2001; Niladevi and Prema, 2008).

In our findings, all the lignin model compounds studied (guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid) were capable of inducing peroxidase production by *R. ornithinolytica* OKOH-1. Nevertheless, guaiacol enhanced peroxidase production by approximately 74 % (Fig. 7.4), making it the best inducer of peroxidase production by the test bacteria, which is contrary to the previous study by Musengi *et al.* (2014) where veratryl alcohol was the best inducer of peroxidase production by *Streptomyces* sp. strain BSII#1. However, no comparative data was found on peroxidase production by *Raoultella* species.

The effect of nitrogen sources on ligninolytic enzyme production by various organisms seems to lack consistency (Niladevi and Prema, 2008). Although fermentation media with sufficient nitrogen has enhanced production of ligninolytic enzymes in some fungi (Kaal *et al.,* 1995), production of lignin modifying enzymes by *Phanerochaete chrysosporium*, one of the most studied fungi, has been limited by high nitrogen concentration (Buswell, 1992). The supplementation of yeast extract in the kraft lignin modified fermentation medium with ammonium chloride gave the best peroxidase production yield (Fig. 7.5).

The production of peroxidase and non-peroxide dependent extracellular enzyme by *R. ornithinolytica* OKOH-1 increased significantly at the logarithmic growth phase reaching maximum productivity at 72 h (Fig. 7.6), corresponding to early stationary growth phase. Thereafter, there was a sharp decrease in the enzyme production at 96 h, which perhaps, can be ascribed to depletion of nutrients or proteolytic activities (Papagianni and Moo-Young, 2002; Fatokun *et al.,* 2016). This finding suggests that the production of peroxidase and non-peroxide dependent extracellular enzyme by *R. ornithinolytica* strain OKOH-1 was growth-associated (McCarthy, 1987; Niladevi and Prema, 2008). This finding is in agreement with previous studies where optimum peroxidase production occurred during the early stationary growth phase. Rob *et*

al. (1997) reported maximum peroxidase production by *Streptomyces avermitilis* UAH 30 at 72 h. Also, Tuncer *et al.* (1999) reported 72 h as the maximum incubation time for peroxidase production by *Thermomonospora fusca* BD25. This is further corroborated by Nour El-Dein *et al.* (2014) who recorded maximum peroxidase production by *Streptomyces* sp. K37 at 72 h of incubation.

Recently, we have suggested the exploration of alternative cheap sources of carbon for fermentation as an important strategy to reduce the cost of enzyme production (Falade *et al.,* 2017a). The use of lignocellulosic biomass as cheap sources of carbon has been advocated due to its abundance, availability and renewable nature (Falade *et al.* 2017a). Valorization of various lignocellulosic wastes including rice straw, wheat straw, sawdust, pea pods etc. for the production of different lignocellulolytic enzymes has been reported (Knezevic *et al.,* 2013; Saratale *et al.,* 2014; Sharma *et al.,* 2015; Neifar *et al.,* 2016).

SSF seems to be the most appropriate fermentation technique for optimum valorization of lignocellulosic wastes for enzyme production. Moreover, enzyme production by bacteria under SSF is said to be economical (Muthukumarasamy and Murugan, 2014). SSF also presents some advantages such as higher production yield, lower wastewater output and reduced energy demand over submerged fermentation (Pandey *et al.,* 2001; Niladevi *et al.,* 2007). In this study, we valorized sawdust, wheat straw and corn stover as solid substrates for peroxidase production under SSF. Of all the lignocellulosic wastes tested, sawdust seemed to be the most promising substrate for peroxidase production by *R. ornithinolytica* OKOH-1, with specific productivity of 15.21 ± 2.48 U mg^{-1} protein (Table 7.1). The high peroxidase production observed on sawdust could be attributed to the inductive effect of its phenolic and non-phenolic components while the production of non-peroxide dependent extracellular enzyme which suggests probable laccase production on sawdust might be related to its phenolic compounds. This finding agrees with that of Knezevic *et al.* (2013) who reported the production of manganese peroxidase and laccase on sawdust. This is further corroborated by Kamsani *et al.* (2016) who also reported high yields of manganese peroxidase and laccase by Bacillus species grown on sawdust under SSF. These findings indicate that sawdust could be used as a cheap renewable substrate for peroxidase production by *R. ornithinolytica* OKOH-1, thus consequently reducing the high cost of enzyme production as well as serving as a waste management strategy.

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CHAPTER EIGHT

Biochemical and molecular characterization of *Raoultella ornithinolytica* peroxidase with biotechnological potentials in dye decolourization and development of cosmetic agent

(*Submitted to Biochimie*)

Abstract

The increase in industrial demand for peroxidases has necessitated the search for novel peroxidase with versatility and catalytic efficiency. Crude peroxidase produced by *Raoultella ornithinolytica* OKOH- 1 (KX640917) was therefore characterized using biochemical and molecular approaches. Subsequently, the enzyme was evaluated for its dye decolourization potential. *R. ornithinolytica* OKOH- 1 peroxidase (RaoPrx) was capable of oxidizing various substrates with pyrogallol giving the optimum activity (K_m: 3.80 mmol L^{-1}, V_{max}: 4.65 µmol mL^{-1} min^{-1}). RaoPrx had an optimum activity at pH 6 and was stable over a pH range of 5.0-7.0 with residual activity of above 40 % after 120 min of incubation. The enzyme showed an optimum activity at 50 ^oC and was very stable at higher temperatures (50 – 70 ^oC) with residual activity of above 70 % after 120 min. The activity of the enzyme was remarkably stable at 50 ^oC as it retained over 90 % of its original activity after 120 min. Moreover, the peroxidase activity was significantly enhanced by Ag^+, $Cu^{2+,}$ $Zn^{2+}$and Fe^{2+} while it was inhibited by $Ca^{2+,}$ $Mg^{2+,}$ $Ba^{2+,}$ Al^{3+}, Co^{2+}, NaN_3 and EDTA with a dissociation constant (K_i) of 0.83 mmol L^{-1} for $CaCl_2$ (10 mmol L^{-1}). Furthermore, characterization of the peroxidase gene suggests it encodes a novel DyP-type peroxidase with mo1ecular weight of 17.587 kDa and isoelectric point of 4.51. RaoPrx exhibited a remarkable dye-decolourizing activity on congo red (65.03%) and melanin (47.96 %) within 30 min. This indicates the potentiality of RaoPrx for applications in dye decolourization and development of cosmetic agent.

Keywords: DyP-type peroxidase, enzyme characterization, enzyme kinetics, thermostability, polymerase chain reaction, peroxidase gene.

8.1. Introduction

R. ornithinolytica OKOH-1 is a new gamma-proteobacteria strain with enormous biotechnological potentials as some microbes belonging to Raoultella species have been implicated in the production of biomolecules of industrial significance such as pullulanase, a debranching enzyme hydrolyzing pullulan and branched polysaccharides (Hii *et al.,* 2012), polysaccharide-protein complex and tri-peptide complex (Fiolka *et al.,* 2013; 2015). Worthy of note, is the production of 2,3-Butanediol (2,3 BD) by *R. ornithinolytica* S12, as an alternative to the petroleum-based 2,3 BD production (Kim *et al.,* 2016, 2017). More so, Raoultella species have recently been implicated in lignin-degradation activities (Bao *et al.,* 2015; Kameshwar and Qin, 2016). Besides, our preliminary study has identified *R. ornithinolytica* OKOH-1 as a ligninolytic bacteria with an excellent peroxidase production potential (Falade *et al.,* 2017b). However, there is dearth of information on characterization of peroxidase from *R. ornithinolytica* in the literature.

Peroxidases (EC. 1.11.1) are ubiquitous as they are widely distributed in eukaryotes and prokaryotes (Battistuzzi *et al.,* 2010). They are broadly divided into heme and non-heme peroxidases, with the heme-containing peroxidases as the most abundant in nature (Zamocky and Obinger, 2010). Various biotechnological potentials of peroxidases have been reported (Falade *et al.,* 2017a). The industrial application potentials of peroxidases have been attributed to their high redox potential for oxidation of recalcitrant compounds (Falade *et al.,* 2017a) including synthetic dyes, whose discharge as industrial effluent can lead to serious environmental pollution problems (Yanto *et al.,* 2014). Considering the danger posed by textile dyes in the environment; and their toxicity, their removal from the environment is of utmost priority.

The involvement of microbial enzymes (peroxidases, laccases and azo reductase) in biodegradation of dyes has been advocated (Kalyani *et al.*, 2011). The application of peroxidases for decolourization of a range of structurally different dyes has been reported (Ollikka *et al.*, 1993; Ferreira-Leitao *et al.*, 2007; Parshetti *et al.*, 2012). Moreover, crude and purified forms of peroxidases have both been used in dye decolourization (Dawkar *et al.,* 2009; Ghodake *et al.,* 2009). It is therefore clear that peroxidases hold a great potential for effective dye removal from the environment.

Besides the potential of peroxidases in bioremediation of textile dyes, decolourization of melanin, the dark pigment responsible for human skin and hair colouration, is desirable as it holds a great

potential in the development of skin-lightening agents. The ability of peroxidases, to oxidize a wide range of structurally different substrates makes them suitable candidates for the oxidation of melanin, which is structurally similar to lignin. Furthermore, the potential of peroxidases for melanin decolourization as well as their prospects in the development of skin lightening agents have been reported (Woo *et al.*, 2004; Nagasaki *et al.*, 2008; Falade *et al.*, 2017a).

The increased utility of peroxidases in various industrial sectors and consequent increase in demand have necessitated the search for novel peroxidase with excellent industrial versatility and catalytic efficiency. Therefore, this study aimed at characterizing peroxidase from *R. ornithinolytica* OKOH-1 using biochemical and molecular approaches.

8.2. Materials and methods

8.2.1. Materials

Hydrogen peroxide, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, yeast extract, remazol brilliant blue R, congo red, ethylenediaminetetraacetic acid (EDTA), sodium azide (NaN_3), magnesium sulphate, ammonium chloride and all other metallic salts used in this study were sourced from Merck KGaA, Darmstadt, Germany while 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), guaiacol, veratryl alcohol, pyrogallol, reactive blue 4 , melanin and kraft lignin were products of Sigma-Aldrich, South Africa. All other chemicals are of analytical grade.

8.2.2. Microorganism

The microorganism used is a new lignin-degrading gamma-proteobacteria, *R. ornithinolytic* OKOH-1 (KX640917) isolated from the sediment of Tyhume River in Alice, Eastern Cape Province, South Africa. The organism was maintained at 4 ^{o}C on MM-L (minimal salt medium supplemented with 1 g L^{-1} of kraft lignin) agar slant.

8.2.3. Enzyme production and preparation

Crude peroxidase was produced and prepared as earlier described by Falade *et al.* (2017b) using submerged fermentation, but with slight modifications. Briefly, the bacterial culture was incubated at 35 ^{o}C and 150 rpm for 72 h. Thereafter, the culture was harvested by centrifugation at 15000

rpm for 10 min at 4 ^{o}C. The recovered supernatant was then utilized as crude enzyme for subsequent analysis.

8.2.4. Determination of peroxidase activity

The peroxidase activity of the recovered supernatant was determined using the method of Chance and Maehly (1955) with minor modifications as previously described by Falade *et al.* (2017b).

8.2.5. Substrate specificity and kinetic properties of peroxidase from R. ornithinolytica OKOH-1 (RaoPrx)

Substrate specificity of the crude enzyme was determined using ABTS: 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), veratryl alcohol, guaiacol, pyrogallol and 2, 6-dimethoxyphenol (2, 6-DMP) as a substrate in 100 mmol L^{-1} potassium phosphate and sodium tartrate buffers as appropriate. Activities against the substrates were assayed at 2 mmol L^{-1}. Subsequently, the kinetic properties were determined by using varied concentrations of pyrogallol (2 mmol L^{-1} to 80 mmol L^{-1}) and a fixed concentration of hydrogen peroxide (4 mmol L^{-1}) at pH 6 (100 mmol L^{-1} potassium phosphate buffer) and 25 ^{o}C. The substrate binding constant (K_m) and maximum rate (V_{max}) were extrapolated from the Michaelis-Menten curve constructed by plotting the rate of purpurogallin formation (V) against pyrogallol concentrations [S].

8.2.6. Biochemical characterization of RaoPrx

8.2.6.1. Effect of pH on activity and stability of RaoPrx

The effect of pH on peroxidase activity was determined within a pH range of 3-7 by using pyrogallol as a substrate. Briefly, the crude enzyme was incubated with 5 % w/v pyrogallol prepared in the different buffers (pH 3-7): 100 mmol L^{-1} sodium tartrate buffer (pH 3-5) and 100 mmol L^{-1} potassium phosphate buffer (pH 6 & 7). The peroxidase activity was determined using standard assay procedure earlier described. In determining the effect of pH on stability of peroxidase, the enzyme was incubated in appropriate buffers (pH 5-7) excluding the substrate for 120 min at room temperature. Subsequently, the residual peroxidase activity was determined using standard assay procedure.

8.2.6.2. Effect of temperature on activity and stability of RaoPrx

The effect of temperature on peroxidase activity was determined by incubating the reaction mixture at temperatures ranging from 30 to 60 ^{o}C under standard assay conditions in the SynergyMx 96-well microtitre plate reader (BioTeK Instruments). However, the thermal stability of the crude peroxidase was determined by incubating the enzyme excluding the substrate at temperatures ranging from 30 to 70 ^{o}C for 120 min in a Dri Block DB-BD (TECHNE, Lasec, SA). Thereafter, the residual peroxidase activity was determined using the earlier described assay procedure.

8.2.6.3. Effect of metal ions and possible inhibitors on activity of RaoPrx

The effects of selected metal ions **(**Ag^{+}, $Cu^{2+,}$ Zn^{2+}, Fe^{2+}, $Ca^{2+,}$ $Mg^{2+,}$ $Ba^{2+,}$ Co^{2+} and Al^{3+}) and EDTA on peroxidase activity were investigated by adding 1 mmol L^{-1} and 10 mmol L^{-1} of each metallic salt (AgCl, $CuCl_2$, $ZnCl_2$, $FeSO_4$, $CaCl_2$, $MgCl_2$, $BaCl_2$, $CoCl_2$, $AlCl_3$) and possible inhibitors (EDTA and NaN_3) to the reaction mixture. Peroxidase activity was thereafter determined according to standard assay procedure.

8.2.7. RaoPrx inhibition kinetics

The kinetics of peroxidase inhibition was studied by using Calcium chloride ($CaCl_2$). Briefly, 10 mmol L^{-1} of $CaCl_2$ was added to the reaction mixture containing the crude enzyme, pyrogallol at varied concentrations (2 mmol L^{-1} to 10 mmol L^{-1}) and 100 mmol L^{-1} potassium phosphate buffer (pH 6). The reaction was initiated by the addition of 0.5 % hydrogen peroxide (30% w/w). Peroxidase activity was then determined as previously described. Control experiment was run in parallel without the inhibitor under the same assay conditions. The dissociation constant, K_i was determined using the modified Lineweaver-Burke equation:

$K_i = K_m [I]/(K_m, \text{apparent} – K_m)$ ……..Eqn. 1.

K_m = Substrate binding constant in the absence of inhibitor

K_m, apparent = Substrate binding constant in the presence of inhibitor

[I] = Concentration of inhibitor.

K_m and K_m, apparent were determined from the plot of 1/V against 1/[S], where V is the rate of purpurogallin formation and [S] is the concentration of pyrogallol. The degree of affinity of the inhibitor with the enzyme was subsequently measured by K_i/K_m.

8.2.8. Molecular characterization of RaoPrx and detection of multicopper oxidase (MCO) gene

8.2.8.1. DNA extraction

Genomic DNA was extracted from the bacterium using boiling method as described by Maugeri *et al.* (2006). A number of colonies of the organism were suspended in 200 µL of nuclease-free water and heated at 100 ^{o}C for 10 min using Dri Block DB-BD (TECHNE, Lasec, SA). Thereafter, the mixture was centrifuged at 20000 x g for 5 min (HERMLE Z 233 M-2, Lasec, SA) and the recovered supernatant was used as DNA template for polymerase chain reaction.

8.2.8.2. Polymerase chain reaction (PCR)

The target genes were amplified using the sets of primers listed in Table 8.1 in a conventional PCR assay. The oligonucleotide primers (Raoprx F and Raoprx R for peroxidase gene and Raomco F and Raomco R for multicopper oxidase gene) which were newly designed for this study, were synthesized by Inqaba Biotech, South Africa. A total of 25 µL reaction mixture which comprised 12.5 µL of master mix (BioLabs, SA), 1 µL each of both forward and reverse primers, 5.5 µL of nuclease-free water and 5 µL of extracted DNA was used for the assay in a PCR thermocycler apparatus (G-STORM, UK) under the following optimized conditions: initial denaturation at 95 ^{o}C (5 min), denaturation at 94 ^{o}C (1 min), annealing at 58 ^{o}C (1 min), extension at 72 ^{o}C (1 min) for 35 cycles and final extension at 72 ^{o}C (5 min). Subsequently, the amplified products were subjected to electrophoresis in 1.5 % agarose gel (Merck, SA), which was visualized in ethidium bromide (Sigma-Aldrich, SA) staining with the use of ultraviolet trans-illuminator (Alliance 4.7, France).

Table 8.1. List of primers for peroxidase and multicopper oxidase genes in *R. ornithinolytica*

Primer name	**Primer sequence (5'-3')**	**Target gene**	**Expected band size**	**Reference**
Raoprx F	AAGGCAGGCTCTGACGAACAA	***Prx***	543 bp	This study
Raoprx R	TGGTGGCTTTTGGCAATAACG			
Raomco F	TCATCTGCCCCTTGTCGCTC	***Mco***	528 bp	This study
Raomco R	GCTGGCTTCGCTTGCGTTTA			

8.2.8.3. Sanger sequencing analysis

The amplified products were analyzed using Sanger dideoxy sequencing method. Prior to sequencing analysis, the PCR products were purified using ExoSAP (Exonuclease-Shrimp Alkaline Phosphatase). The ExoSAP master mix was prepared by adding 50 µL of Exonuclease I (NEB M0293) 20 U μL^{-1} to 200 µL of Shrimp Alkaline Phosphatase (NEB M0371) 1 U μL^{-1} in a 600 µL micro-centrifuge tube. Thereafter, 2.5 µL of the ExoSAP mix was added to 10 µL of the PCR product. The mixture was then incubated at 37 ^{o}C for 30 min followed by heating at 95 ^{o}C for 5 min to terminate the reaction. Subsequently, the purified PCR products were sequenced using the ABI V3.1 Big dye kit according to the manufacturer's instructions on ABI3500XL genetic analyser, with a 50cm array. The sequencing products were further purified with the Zymo Seq clean up kit (Zymo Research) and analyzed using main work bench 7 followed by a BLAST search in UniProt Knowledgebase (www.uniprot.org) and PeroxiBase database (peroxibase.toulouse.inra.fr) using Blastx program.

8.2.8.4. Phylogenetic analysis

The phylogenetic analysis of the translated protein sequence was conducted in MEGA 7.0.21 (Kumar *et al.,* 2016) using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis (Sneath and Sokal, 1973) while the disparity index for all sequence pairs was determined using the model of Kumar and Gadagkar (2001). Multiple sequence alignment was conducted using CLUSTAL W in BioEdit sequence alignment editor (Thompson *et al.*, 1994) as the physicochemical properties of the deduced partial amino acids were determined using GENEIOUS 10.2.2.

8.2.9. Dye decolourization study

The dye decolourization potential of RaoPrx was evaluated using selected synthetic dyes (congo red: CR, remazol brilliant blue R: RBBR, reactive blue 4: RB4 and melanin). The rate of dye decolourization was determined in line with the modified method of Kalyani *et al.* (2011). The reaction mixture contained dye (100 mg L^{-1}), potassium phosphate buffer (100 mmol L^{-1}, pH 6) and culture supernatant (crude enzyme). The reaction was initiated via the addition of 0.5% hydrogen peroxide (30 % w/w) and subsequent incubation at predetermined optimum temperature for 30 min (CR, RBBR, RB4) and 20 min for melanin. Absorbance was read at 490 nm, 590 nm,

595 nm and 475 nm respectively (Woo *et al.*, 2004; Kalyani *et al.*, 2011). The reaction mixture without the crude enzyme served as the control. Dye decolourization was measured by monitoring the decrease in absorbance of each dye and expressed as percentage decolourization:

$$\frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \times 100\% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\text{Eqn. 2.}$$

8.2.10. Data analysis

Where applicable, data were subjected to analysis of variance (ANOVA) using GraphPad Prism 7 at a 5% ($P \leq 0.05$) confidence interval. Results were presented as mean values ± standard deviation (STD).

8.3. Results and discussion

8.3.1. Substrate specificity and kinetic properties of RaoPrx

The results of substrate specificity by RaoPrx as presented in Table 8.2 showed that peroxidase from *R. ornithinolytica* OKOH-1 had activity on all the substrates (ABTS, veratryl alcohol, guaiacol and pyrogallol) except 2,6-DMP, with the highest peroxidase activity exhibited on pyrogallol. This finding indicates that peroxidase from *R. ornithinolytica* OKOH-1 had a wide substrate specificity, with the highest affinity for pyrogallol, which was subsequently used as the enzyme substrate throughout the study. More so, the oxidation of pyrogallol by RaoPrx is consistent with Michaelis-Menten equation as revealed in Fig. 8.1 which showed that RaoPrx had a K_m of about 3.8 mmol L^{-1} and V_{max} of 4.65 µmol mL^{-1} min^{-1} on pyrogallol.

Table 8.2. Substrate specificity of peroxidase from *R. ornithinolytica* OKOH-1

Substrate	Assay conditions	Wave length	Relative peroxidase activity (%)	Reference
ABTS	2 mmol L^{-1} ABTS; 4 mmol L^{-1} H_2O_2; 100 mmol L^{-1} potassium phosphate buffer, pH 6.	420	0.35	Mongkolthanaruk *et al.* (2012)
Veratryl alcohol	2 mmol L^{-1} veratryl alcohol; 4 mmol L^{-1} H_2O_2; 100 mmol L^{-1} sodium tartrate buffer, pH 3	310	3.40	Tien and Kirk (1988)
Guaiacol	2 mmol L^{-1} guaiacol; 4 mmol L^{-1} H_2O_2; 100 mmol L^{-1} sodium tartrate buffer, pH 5	465	0.17	Paszczynski *et al.* (1988)
Pyrogallol	2 mmol L^{-1} pyrogallol; 4 mmol L^{-1} H_2O_2; 100 mmol L^{-1} potassium phosphate buffer, pH 6	420	100	Chance and Maehly (1955)
2, 6-DMP	2 mmol L^{-1} 2,6-DMP; 4 mmol L^{-1} H_2O_2; 100 mmol L^{-1} sodium tartrate buffer, pH 3	469	0	Perez-Boada *et al.* (2002)

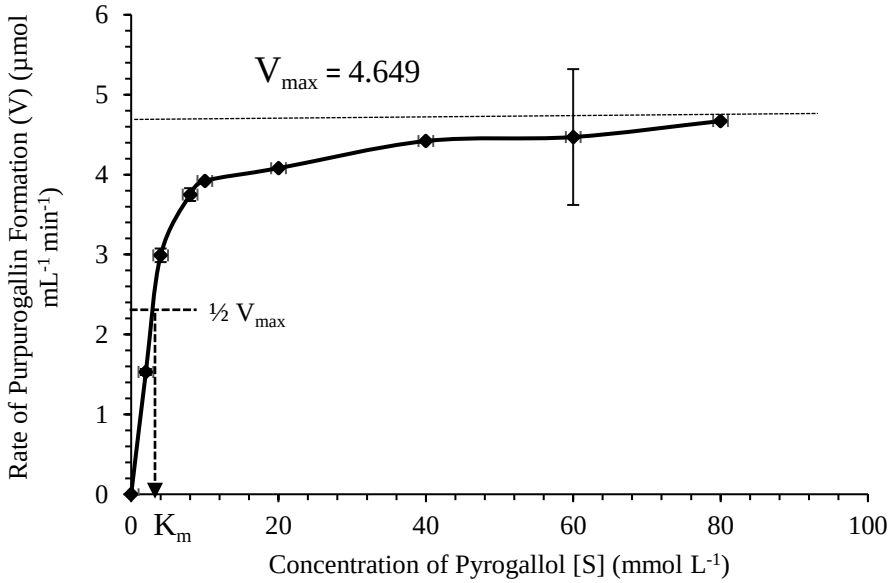

Fig. 8.1. Michaelis-menten kinetics of peroxidase activity using pyrogallol as substrate.

8.3.2. Effect of pH on activity and stability of RaoPrx

The activity of peroxidase from *R. ornithinolytica* OKOH-1 was determined at pH ranging from 3.0 to 7.0. The results revealed that the enzyme had optimum activity at pH 6.0 (Fig. 8.2a) with relative activity of 47.3 % at pH 7.0 and 37.8 % at pH 5.0. However, no peroxidase activity was detected at pH 3.0 and 4.0. This finding is comparable to the result of Fodil *et al.* (2011) who reported optimum activity for a peroxidase produced by *Streptomyces* sp. strain AM2 at pH 6.0 On the other hand, Olajuyigbe *et al.* (2015) recorded optimum activity for crude peroxidase from *Actinomyces viscosus* at pH 7.0 while Casciello *et al.* (2017) reported optimum activity for *Nonomuraea gerezanensis* peroxidase at pH 4. The enzyme exhibited stability over a short pH range of 5.0-7.0. after 120 min of incubation. It is most stable at pH 6.0. as it retained 96 % of its original activity after 30 min and more than 50 % after 120 min at this pH. The enzyme had residual activity of 46.4 % and 56.7 % at pH 5.0 and pH 7.0 respectively after 120 min (Fig. 8.2b). This finding is consistent with previous related studies where peroxidase was stable within a short pH range as observed in this study. For instance, Fodil *et al.* (2012) reported peroxidase stability at a pH range of 4.0-8.0 while Olajuyigbe *et al.* (2015) and Casciello *et al.* (2017) recorded peroxidase stability within the pH range of 6.0-8.0 and 4.0-5.0, respectively.

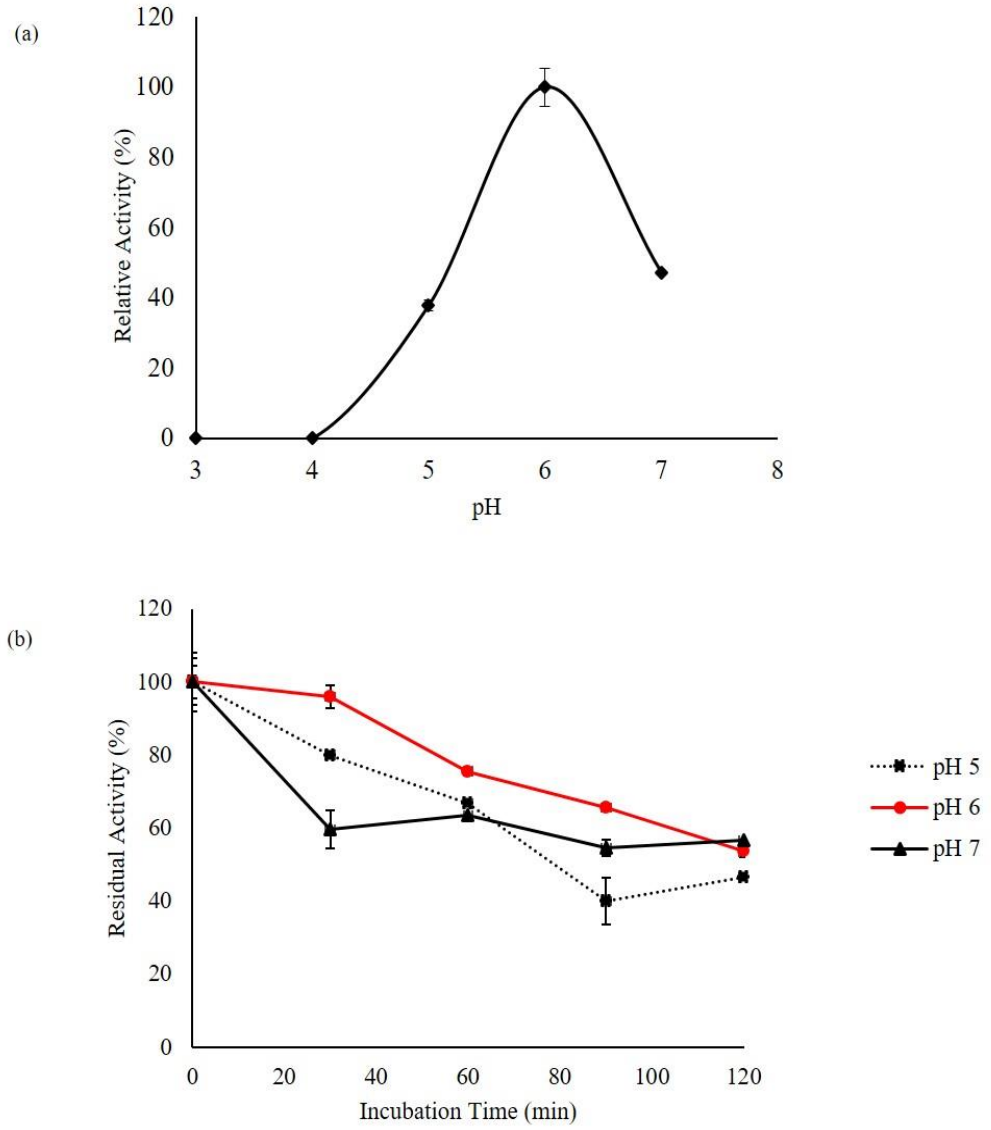

Fig. 8.2. Effect of pH on activity and stability of RaoPrx. (a). Effect of pH on *R. ornithinolytica* peroxidase activity. (b). Effect of pH on *R. ornithinolytica* peroxidase stability (Error bars indicate mean ± standard deviation of triplicate values).

8.3.3. Effect of temperature on activity and stability of RaoPrx

The activity of RaoPrx was determined using the temperature range of 30 to 60 ^{o}C. *R. ornithinolytica* peroxidase had optimum activity at 50 ^{o}C but was active over the temperature range used, with relative activity of 43.6 %, 49.6 % and 32.9 % at 30 ^{o}C, 40 ^{o}C and 60 ^{o}C respectively (Fig. 8.3a). The sharp decrease in peroxidase activity observed at 60 ^{o}C, is perhaps due to thermal denaturation that likely occurred as a result of increase in chemical potential energy capable of altering the three-dimensional structure of the protein while the steady increase in relative peroxidase activity from 30 to 50 ^{o}C may be attributed to the increase in kinetic energy and number of collisions of enzyme and substrate per unit time. The optimum temperature for peroxidase recorded in this study is comparable to what had previously been reported (Fodil *et al.,* 2011; Olajuyigbe *et al.,* 2015). However, Kalyani *et al.* (2011) reported 40 ^{o}C as optimum temperature for peroxidase from *Pseudomonas* sp. SUK 1 while Fodil *et al.* (2012) and Casciello *et al.* (2017) reported a higher optimum temperature of 80 ^{o}C and 60 ^{o}C respectively. Interestingly, RaoPrx was very stable at higher temperatures (50 – 70^{o}C) with residual activity of over 70 % after 120 min (Fig. 8.3b). The activity of the enzyme was remarkably stable at 50 ^{o}C as it retained 93.5 % of its original activity after 120 min. It is worthy of note, that RaoPrx has a higher thermostability than some microbial peroxidases previously reported. For instance, Olajuyigbe *et al.* (2015) reported residual activity of 60 % at 40 ^{o}C and 50 ^{o}C after 60 min for peroxidases from two different actinomyces species, which completely lost their activities after 150 min at 70 ^{o}C. More so, peroxidase from *Rhizoctonia* sp. SYBC-M3 was not stable at temperatures above 50 ^{o}C (Cai *et al.,* 2010) while *Phanerochaete chrysosporium* peroxidase completely lost its activity at 65 ^{o}C after 60 min (Urek and Pazarlioglu, 2004). This finding indicates that the peroxidase from *R. ornithinolytica* OKOH-1 is thermostable and this augurs well for biotechnological applications.

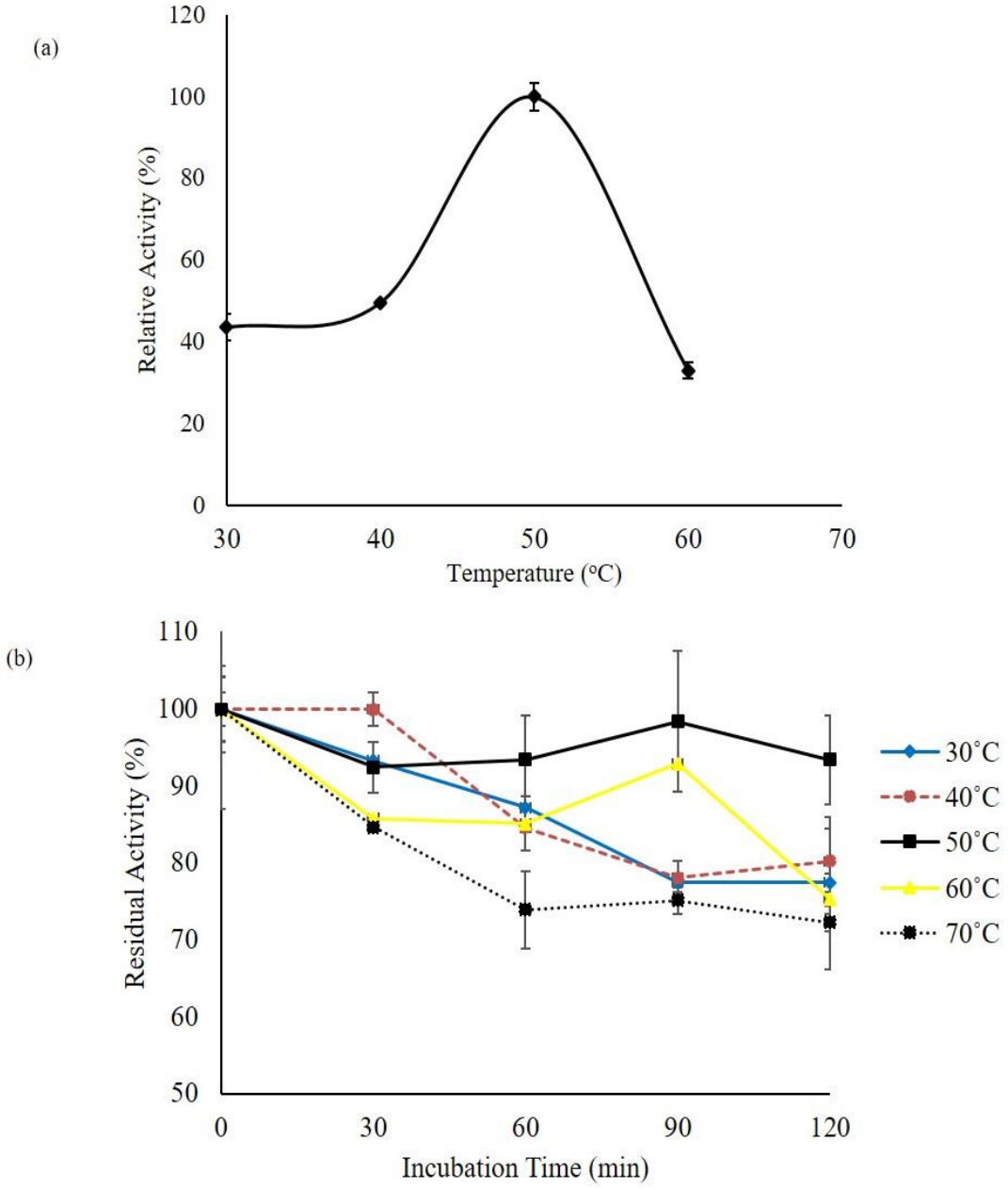

Fig. 8.3. Effect of temperature on activity and stability of RaoPrx (a). Effect of temperature on *R. ornithinolytica* peroxidase activity. (b). Effect of temperature on *R. ornithinolytica* peroxidase stability (Error bars indicate mean ± standard deviation of triplicate values).

8.3.4. Effect of metal ions and possible inhibitors on activity of RaoPrx

The activity of peroxidase from *R. ornithinolytica* OKOH-1 was significantly enhanced in the presence of both low (1 mmol L^{-1}) and high (10 mmol L^{-1}) concentrations of Ag^{+}, $Cu^{2+,}$ $Zn^{2+}$and Fe^{2+} when compared with the control (Table 8.3), with Cu^{2+} having the highest relative activity of 241.68 % and 597.79 % at low and high concentrations, respectively, followed by Fe^{2+} (1 mmol L^{-1}: 126.74 %, 10 mmol L^{-1}: 453.88 %). The increased peroxidase activity observed in the presence of Ag^{+}, $Cu^{2+,}$ $Zn^{2+}$and Fe^{2+} may be attributable to some conformational changes that probably occurred consequent upon the binding of the metal ions on some amino acid residues in the catalytic site of the enzyme (Olajuyigbe and Ogunyewo, 2016). However, the enzyme activity was partially inhibited by $Ca^{2+,}$ $Mg^{2+,}$ $Ba^{2+,}$ Al^{3+} at both low and high concentrations, with relative activities of 45.13 %, 67.08 %, 59.08 % and 56.01 % at 10 mmol L^{-1}, respectively when compared with the control, whereas NaN_3, only inhibited the peroxidase activity at high concentration, with relative activity of 58.25 %. However, the activity of the enzyme was completely inhibited by Co^{2+} and EDTA even at low concentration. This finding is consistent with previous related studies where significant inhibition of peroxidase activity by EDTA had been reported (Asgher *et al.,* 2012; Praveen *et al.,* 2012; Olajuyigbe *et al.,* 2015). Likewise, Fodil *et al.* (2011) reported the inhibitory effect of NaN_3 on peroxidases from *Streptomyces* sp. AM2. The complete inhibition of the enzyme by EDTA might be attributed to its metal chelating activity which rendered the cofactors of peroxidase unavailable for catalytic reaction. This suggests that peroxidase from *R. ornithinolytica* OKOH-1 may depend on a heme component for its catalytic activity (Fodil *et al.,* 2011). Moreover, complete inhibition of RaoPrx activity by Co^{2+} is perhaps due to the ability of cobalt ions to form complexes, which is typical of transition metals or it might be that cobalt (II) reacted with hydrogen peroxide (Atabey *et al.,* 1996), the peroxidase activator, thereby making it unavailable to activate the enzyme appropriately. Furthermore, the kinetic study of RaoPrx inhibition in the presence of 10 mmol L^{-1} $CaCl_2$ as presented in Fig. 8.4. revealed the dissociation constant, K_i of 0.83 mmol L^{-1} with 0.22 as the deduced K_i/K_m value, which suggests that the inhibitor ($CaCl_2$) bound to the enzyme with greater affinity, hence, its stronger effect on the kinetics. This finding further indicates that $CaCl_2$ is a competitive inhibitor of *R. ornithinolytica* OKOH-1 peroxidase as it increased the K_m of the enzyme but did not affect its V_{max} (Berg *et al.,* 2002).

Table 8.3. Effect of metal ions and possible inhibitors on the activity of peroxidase from *R. ornithinolytica* OKOH-1

	Relative activity (%)	
	1 Mm	**10 Mm**
Control	100	100
Ag^{+}	109.30 ± 2.71^{d}	110.27 ± 1.84^{d}
Cu^{2+}	241.68 ± 2.48^{a}	597.79 ± 156.25^{a}
Zn^{2+}	148.06 ± 5.83^{b}	184.11 ± 6.82^{c}
Fe^{2+}	126.74 ± 19.09^{b}	453.88 ± 4.46^{b}
Ca^{2+}	56.54 ± 4.14^{h}	45.13 ± 1.17^{g}
Mg^{2+}	70.25 ± 3.17^{g}	67.08 ± 0.00^{e}
Ba^{2+}	78.81 ± 2.40^{f}	59.08 ± 0.58^{f}
Co^{2+}	0	0
Al^{3+}	88.76 ± 5.16^{e}	56.01 ± 6.20^{f}
NaN_3	118.98 ± 1.24^{c}	58.25 ± 0.00^{f}
EDTA	0	0

Values represent mean ± standard deviation, number of replicate, n = 3. Values with the same superscript letter along the same column are not significantly different ($P > 0.05$).

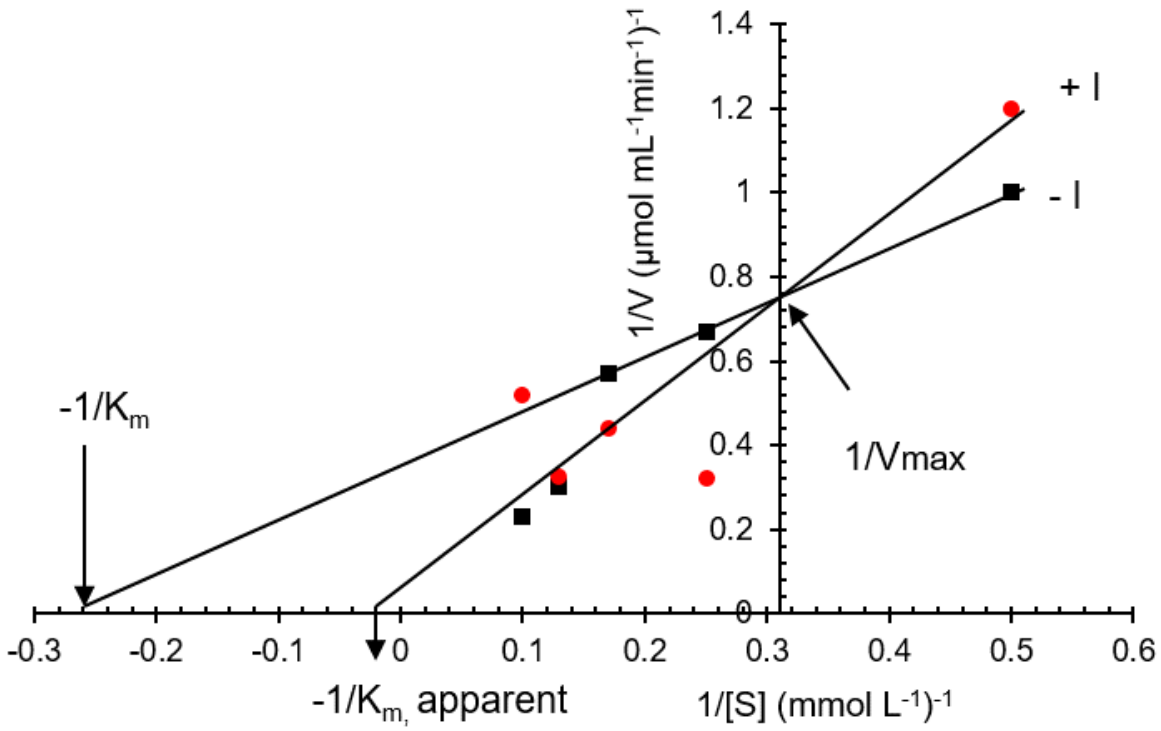

Fig. 8.4. Kinetic study of RaoPrx inhibition by $CaCl_2$. I: Inhibitor ($CaCl_2$), + I: Increasing substrate concentration in the presence of 10 mmol L^{-1} of the inhibitor, - I: Increasing substrate concentration in the absence of the inhibitor.

8.3.5. Molecular characterization of RaoPrx and detection of MCO gene

Fig. 8.5 shows the gel picture of the PCR amplification of peroxidase and MCO genes in *R. ornithinolytica* OKOH-1. The band sizes of the amplified genes corresponded with the expected band sizes of 543 bp and 528 bp respectively. The blast search of the nucleotide sequences of the amplified genes in NCBI database (https://blast.ncbi.nlm.nih.gov) showed 99 % similarities to DyP-type peroxidase (Protein ID: AGJ84824.1) and multicopper oxidase (Protein ID: AGJ87589.1) genes in *R. ornithinolytica* B6 complete genome (GenBank Accession Number: CP004142), respectively. The nucleotide sequences are available in the NCBI database as *Raoultella ornithinolytica* OKOH-1 peroxidase and multicopper oxidase genes, partial cds under the accession numbers MF370527 and MF374335, respectively.

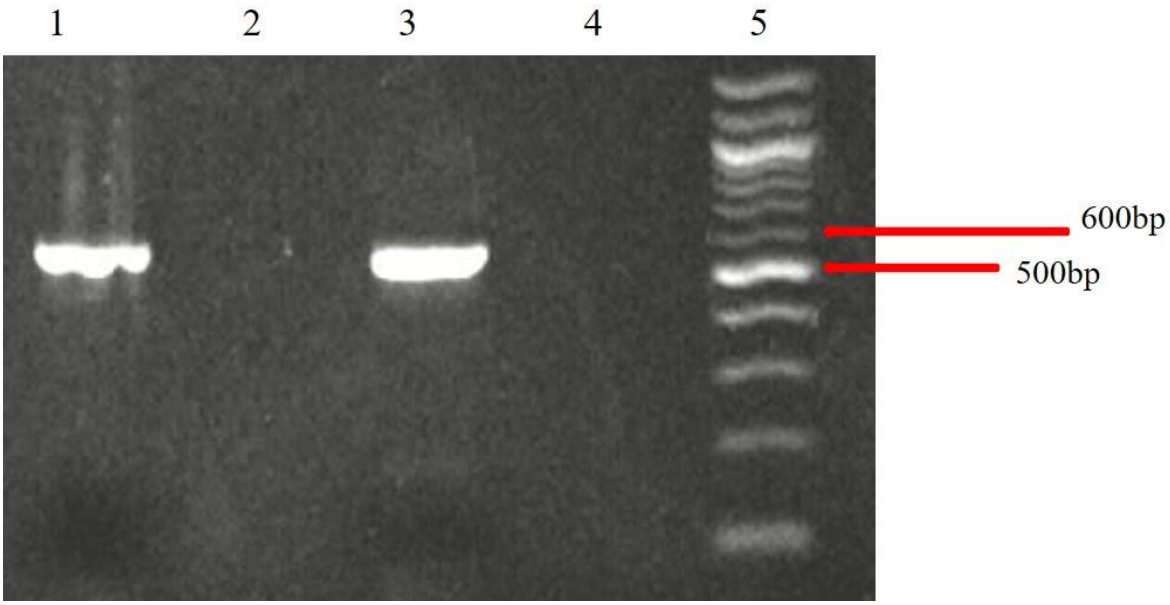

Fig. 8.5. Gel picture of the PCR amplification of peroxidase and multicopper oxidase genes in *R. ornithinolytica* OKOH-1. Lane 1: multicopper oxidase gene (528 bp), lane 3: peroxidase gene (543 bp), lane 5: DNA marker, lanes 2 & 4: negative control for multicopper oxidase and peroxidase genes, respectively.

Multiple alignment of the studied nucleotide sequence with peroxidase gene sequences of other *Raoultella ornithinolytica* in NCBI database (Fig. 8.6) revealed a genetic variation inform of a single nucleotide polymorphism (SNP) at position 5 which makes it unique from other peroxidase genes. The alignment showed that the proposed conserved region for peroxidase in *R. ornithinolytica* comprise of 126 nucleotide sequences with interval span of 145→270 on open reading frame 1 (ORF 1).

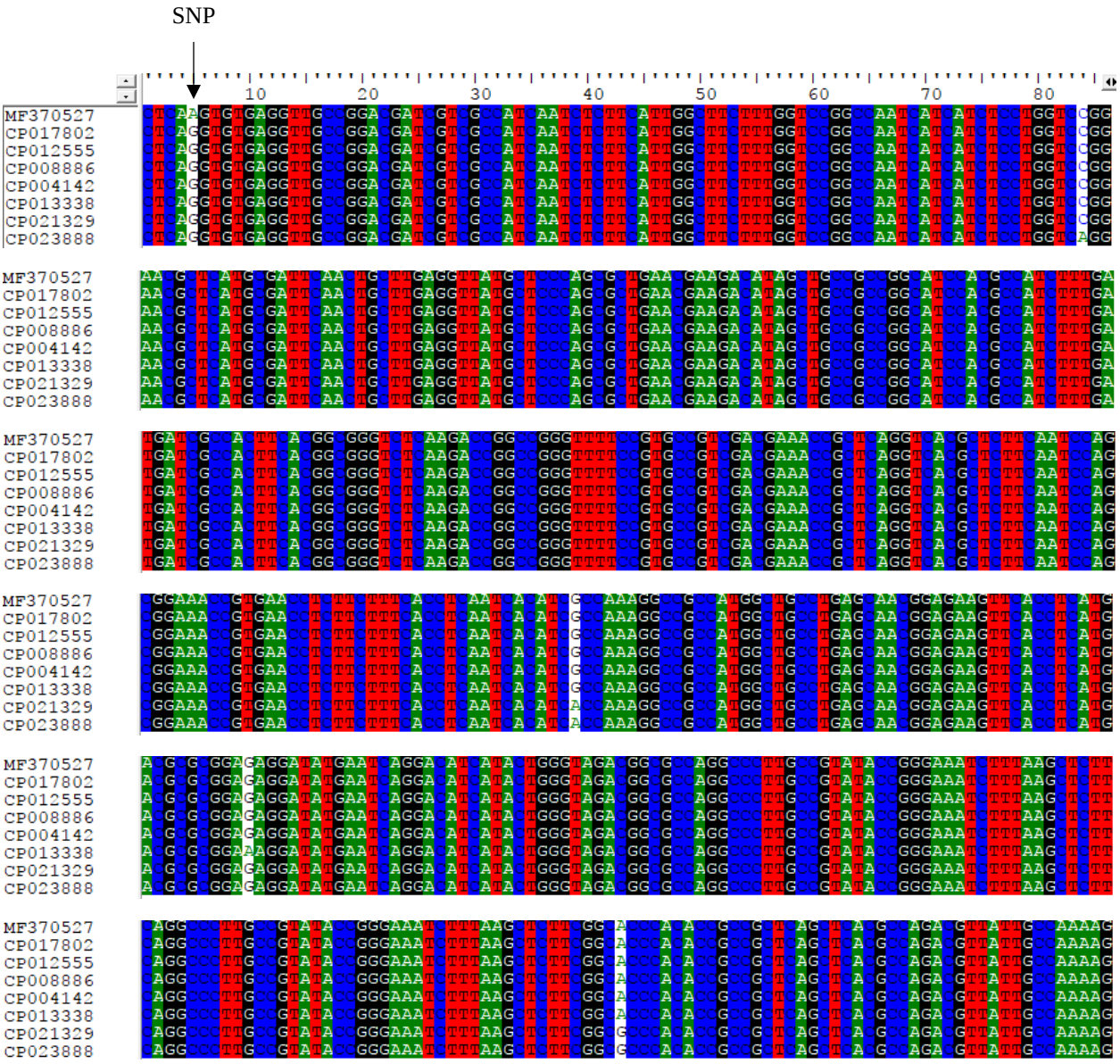

Fig. 8.6. Multiple sequence alignment of peroxidase gene in *R. ornithinolytica.* MF370527: peroxidase gene sequence from this study. Unshaded columns are points of nucleotide variations. SNP: Single Nucleotide Polymorphism.

Furthermore, phylogenetic analysis of the translated protein (GenBank ID: ATO59094.1) and selected DyP-type peroxidases in the peroxidase database: PeroxiBase (peroxibase.toulouse.inra.fr) suggested it belongs to Class B of the DyP-type peroxidase family as it formed a distinct cluster with members of this class (Fig. 8.7). Other classes of bacterial DyP family include A and C while Class D is made up of fungal DyPs (Yoshida and Sugano, 2015). The estimates of net composition bias disparity between amino acid sequences (Table 8.4) revealed 0.00 disparity between *R. ornithinolytica* OKOH-1 peroxidase and most members of Bacterial DyP-type peroxidase family which indicates no evolutionary divergence with RaoPrx.

Further characterization of the amino acid sequence of *R. ornithinolytica* OKOH-1 peroxidase using Geneious 10.2.2 (a bioinformatic analysis tool), estimated a molecular weight (MW) of 17.587 kDa and an isoelectric point of 4.51. Moreover, previous studies had reported different MWs for peroxidases from different bacterial species. Oliveira *et al.* (2009) reported 25 kDa and 40 kDa as the estimated MWs for peroxidase from *Bacillus pumilus* and *Paenibacillus* sp. respectively. Similarly, Fodil *et al.* (2011) reported 25 kDa and 40 kDa as MWs for peroxidases (HaP1 and HaP2, respectively) purified from *Streptomyces* sp. AM2. Nonetheless, HaP3 peroxidase from *Streptomyces* sp. AH4 had an estimated MW of 60 kDa (Fodil *et al.*, 2012) while Ghodake *et al*. (2009) and Kalyani *et al.* (2011) reported 110 kDa and 83 kDa for peroxidases from *Acinetobacter calcoaceticus* NCIM 2890 and *Pseudomonas* sp. SUK 1, respectively.

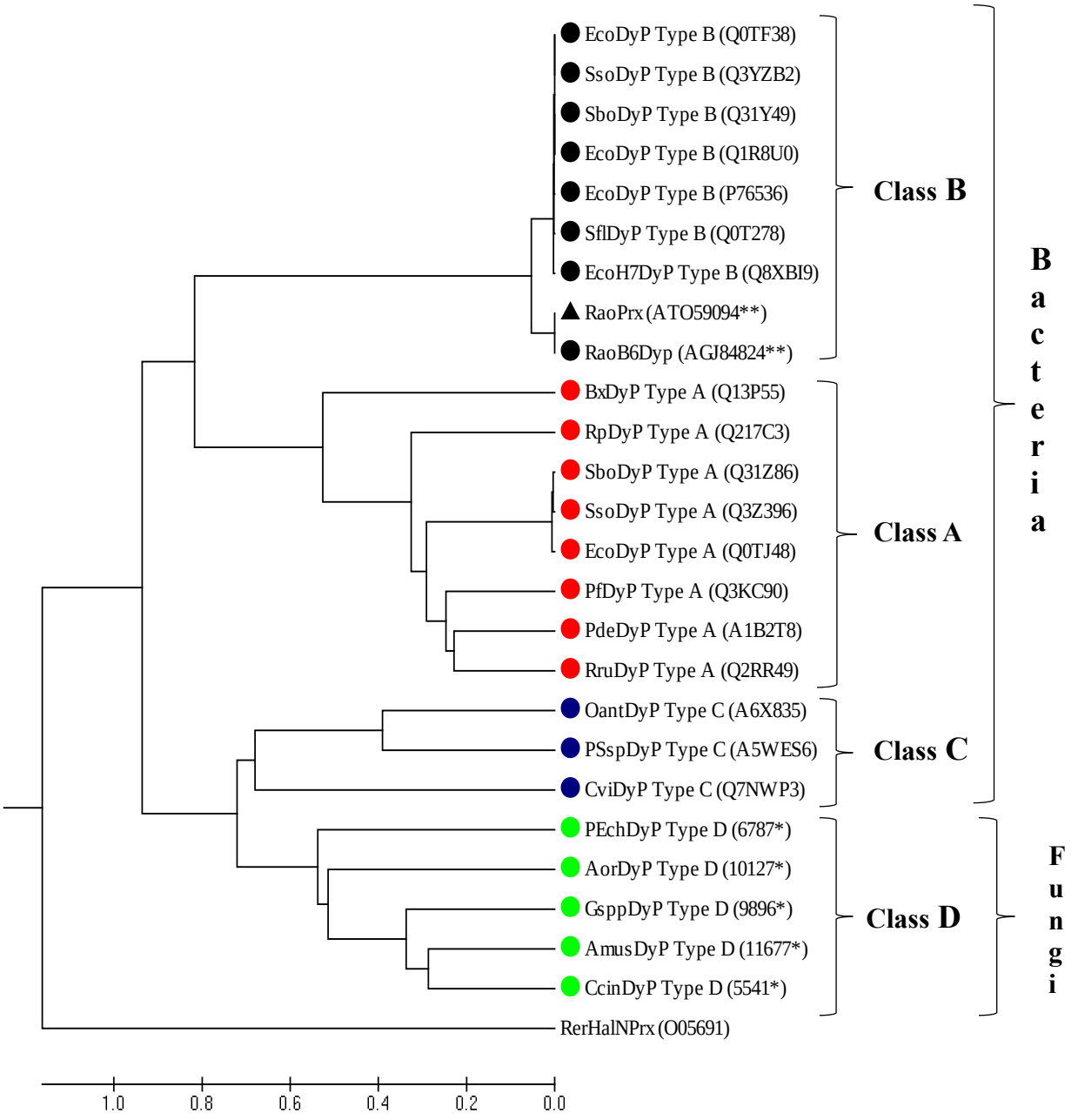

Fig. 8.7. Dendogram of selected DyP-type peroxidases, constructed using UPGMA cluster analysis and poisson correction model. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Red tips indicate DyP-type peroxidase class A, blue tips indicate class C, black tips represent class B while the green tips are for class D. The black triangular tip indicates the studied peroxidase while the one without colour is the outgroup. The UniProtKB reference numbers, GenBank ID** and PeroxiBase ID* of the

proteins are indicated in parentheses. Eco: *Escherichia coli*, Pf: *Pseudomonas fluorescens*, Sbo: *Shigella boydii*, Sso: *Shigella sonnei*, Pde: *Paracoccus denitrificans*, Rp: *Rhodopseudomonas palustris*, Rru: *Rhodospirillum rubrum*, Bx: *Burkholderia xenovorans*, EcoH7: *Escherichia coli* 0157:H7, Sf: *Shigella flexneri*, Oant: *Ochrobactrum anthropic*, PSsp: *Psychrobacter* sp., Cvi: *Chromobacterium violaceum*, Gspp: *Ganoderma* sp., Aor : *Aspergillus oryzae*, Ccin: *Coprinopsis cinereal*, Amus: *Amanita muscaria*, PEch*: Penicillium chrysogenum*, RaoPrx: *Raoultella ornithinolytica* OKOH-1 peroxidase, RaoB6: *Raoultella ornithinolytica* B6, DyP: Dye decolourizing peroxidase. RerHalPrx: *Rhodococcus erythropolis* haloperoxidase.

Moreover, the presence of the gene encoding multicopper oxidase in *R. ornithinolytica* OKOH-1 suggests its potential for expression of laccase activity, which further confirms its biotechnological dexterity. Multicopper oxidases (MCOs) are oxidoreductases that oxidize their substrates with a concomitant four electron reduction of molecular oxygen to water (Sirim *et al.,* 2011). MCOs are classified based on their copper centres: type 1 (blue), type 2 (normal) and type 3 or coupled binuclear (Messerschmidt and Huber, 1990; Ouzounis and Sander, 1991). MCOs is an enzyme family of four, comprising laccases (EC 1.10.3.2), ferroxidases (EC. 1.16.3.1), ascorbate oxidase (EC 1.10.3.3) and ceruloplasmin (EC 1.16.3.1). Laccases, which are the largest member of MCOs, are widely distributed in prokaryotes and eukaryotes (Sirim *et al.,* 2011). The biotechnological potentials of laccases in biopulping, biobleaching, bioremediation, juice/wine clarification, textile dye decolourization, degradation of xenobiotics and effluent treatment have been reported (Couto and Toca Herrera, 2006; Chandra and Chowdhary, 2015; Afreen *et al.,* 2016). Given the enormous industrial application potentials of laccase and increased industrial demand, there is need to explore new sources of laccase with enhanced production. Thus, *R. ornithinolytica* OKOH-1 may be a potential source for laccase production.

Table 8.4. Pattern disparity index between amino acid sequences of *R. ornithinolytica* peroxidase and selected members of bacterial DyP-type peroxidases

RaoPrx (ATO59094)**																			
RaoB6Dyp (AGJ84824**)	0.00																		
EcoDyP Type A (Q0TJ48)	0.00	0.00																	
PfDyP Type A (Q3KC90)	0.00	0.00	0.00																
SboDyP Type A (Q31Z86)	0.00	0.00	0.00	0.00															
SsoDyP Type A (Q3Z396)	0.00	0.00	0.00	0.00	0.00														
PdeDyP Type A (A1B2T8)	0.10	0.10	0.29	0.00	0.33	0.33													
RpDyP Type A (Q217C3)	0.00	0.00	0.06	0.00	0.11	0.11	0.02												
RruDyP Type A (Q2RR49)	0.01	0.01	0.06	0.00	0.07	0.07	0.02	0.18											
BxDyP Type A (Q13P55)	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.01	0.00										
EcoH7DyP Type B (Q8XBI9)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01									
EcoDyP Type B (Q0TF38)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01	0.00								
SboDyP Type B (Q31Y49)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01	0.00	0.00							
SsoDyP Type B (Q3YZB2)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01	0.00	0.00	0.00						
EcoDyP Type B (Q1R8U0)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01	0.00	0.00	0.00	0.00					
SflDyP Type B (Q0T278)	0.00	0.00	0.08	0.00	0.10	0.10	0.37	0.00	0.18	0.01	0.00	0.00	0.00	0.00	0.00				
EcoDyP Type B (P76536)	0.00	0.00	0.10	0.00	0.12	0.12	0.39	0.00	0.20	0.04	0.00	0.00	0.00	0.00	0.00	0.00			
OantDyP Type C (A6X835)	0.00	0.00	0.25	0.00	0.27	0.27	0.00	0.00	0.00	0.00	0.08	0.08	0.08	0.08	0.08	0.08	0.10		
PSspDyP Type C (A5WES6)	0.04	0.04	0.45	0.07	0.41	0.41	0.35	0.51	0.25	0.00	0.30	0.30	0.30	0.30	0.30	0.30	0.33	0.35	
CviDyP Type C (Q7NWP3)	0.00	0.00	0.05	0.00	0.10	0.10	0.00	0.00	0.00	0.00	0.19	0.19	0.19	0.19	0.19	0.19	0.23	0.00	0.38

Values greater than 0 indicate the larger differences in base composition biases than expected based on evolutionary divergence between sequences.

8.3.6. Dye decolourization potential of RaoPrx

The search for effective method of dye decolourization has continued to increase perhaps, due to its health and environmental implications, as well textile dyes have been described as the major sources of environmental pollution (Falade *et al.,* 20017a). Decolourization of synthetic dyes through enzymatic approach had been reported to be effective (Kalyani *et al.,* 2011). Enzymatic decolourization of dyes involves the use of either crude or purified forms of the enzymes (Dawkar *et al.,* 2009; Ghodake *et al.,* 2009). However, considering the cost of enzyme purification, the use of crude enzyme is being encouraged. Therefore, this study evaluated the potential of crude peroxidase from *R. ornithinolytica* OKOH-1 for decolourization of selected synthetic dyes with different aromatic substituent positions (*ortho* and *para*) as the effectiveness of enzyme system in dye decolourization depends on the susceptibility of the arene substituents of the dyes to hydrozylation (Goszczynski *et al.,* 1994). The dye decolourization potential of RaoPrx as presented in Fig. 8.8 showed that the enzyme had maximum decolourization activity of 65.03 % on CR, followed by melanin with 47.96 % while 9.09 % and 4.72 % decolourization was observed on RB4 and RBBR, respectively, within 30 min of incubation.

This finding indicates that the enzyme has the potential for decolourization of a wide range of synthetic dyes. However, the maximum decolourization activity on CR suggests the specificity of *R. ornthinolytica* peroxidase for azo and *ortho* positioned arene substituent dyes. The results further indicate that azo dyes seemed to be more susceptible to decolourization than anthraquinone dyes (RB4 and RBBR) which have their substituents attached at the *para* positions. The discrepancy observed in the rate of dye decolourization by the enzyme might be due to structural variations of the dyes (Murugesan *et al.,* 2006). This finding is in agreement with previous related studies which had also reported different rates of decolourization for different dyes. Kalyani *et al.* (2011) reported decolourization of various textile dyes (Methyl orange, Reactive red 2, Reactive orange 16, Navy Blue HE2R etc.) by *Pseudomonas* sp. SUK 1 peroxidase with decolourization activities of 72 and 45 % on Methyl orange and Reactive orange 16, respectively within 12 h. On the other hand, Rekik *et al.* (2015) reported 5% decolourization of Poly R-478 by peroxidase from *Streptomyces griseosporeus* SN9 after 48 h.

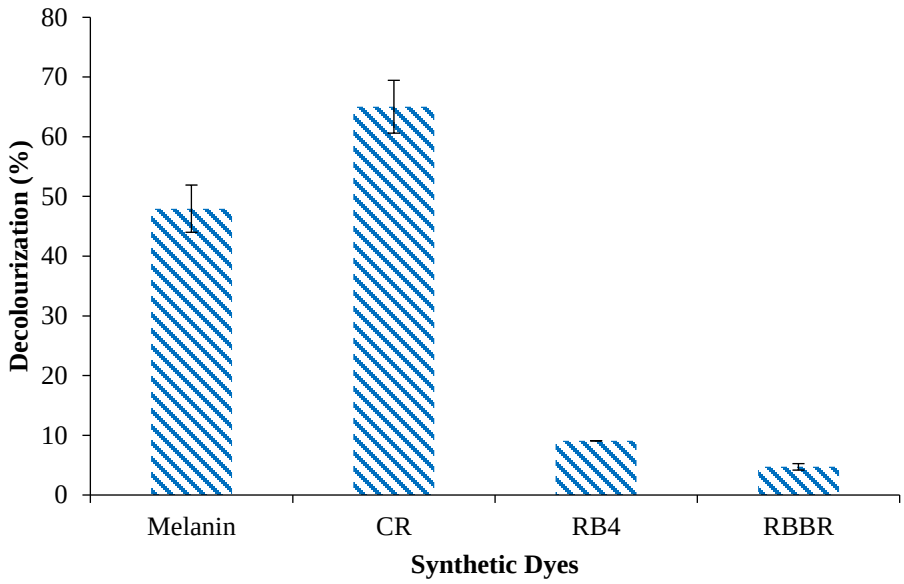

Fig. 8.8. Decolourization of synthetic dyes by peroxidase from *R. ornithinolytica* OKOH-1. CR: Congo Red, RB4: Reactive Blue 4, RBBR: Remazol Brilliant Blue R.

Hydroquinone has been described as the most effective skin-lightening agent (Falade *et al.*, 2017a). However, controversies concerning its safety had led to a search for suitable alternatives. Moreover, the "exploration of alternative agents with the potential to directly decolourize melanin pigment through oxidation" has been advocated (Falade *et al.*, 2017a). Interestingly, *R. ornthinolytica* peroxidase exhibited a decolourization activity of about 48 % on synthetic melanin in just 20 min. This finding indicates the potential of *R. ornthinolytica* peroxidase to serve as an alternative to hydroquinone in the development of skin-lightening agents. This is corroborated by previous studies which had reported the ability of peroxidases to effectively decolourize synthetic melanin (Woo *et al.*, 2004; Nagasaki *et al.*, 2008).

8.4. Conclusion

In conclusion, peroxidase from *R. ornithinolytica* OKOH-1 is a novel thermostable DyP-type peroxidase with biotechnological potentials in textile dye remediation and development of cosmetic agents. More so, the detection of multicopper oxidase gene in *R. ornithinolytica* OKOH-1 suggests its potential for laccase production.

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CHAPTER NINE

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PROSPECTS

9.1. Introduction

This chapter gives a general discussion of the findings of the study under the following headings in line with the specific objectives: ligninolytic bacteria, optimization of environmental and nutritional conditions for peroxidase production, molecular and biochemical characterization of bacterial peroxidases and biotechnological potentials of peroxidases in textile dye remediation and development of cosmetic agents. Also, it gives a conclusion on the study and highlights some new areas of research that could be explored for further studies in the future.

9.2. General discussion

9.2.1. Ligninolytic bacteria

Ligninolytic bacteria are bacteria strains that possess the ability to degrade or mineralize lignin, a recalcitrant constituent of lignocellulosic biomass which confers structural rigidity and support on plant cell walls. Ligninolytic bacteria play a significant role in the conversion of lignocellulosic biomass to value-added products of economic importance such as biofuel as their degradation activities render the saccharide units of cellulose entrapped by the lignin available for fermentation. Thus, they hold a great potential for delignification of feedstocks for biofuel production. As well they are able to utilize the abundant and renewable lignocellulosic materials as cheap and alternative sources of carbon for production of lignin modifying enzymes including peroxidases and laccase.

In this study, a total of 79 presumptive ligninolytic bacteria were isolated based on their ability to grow on alkaline lignin as sole carbon source. Six (6) bacteria strains belonging to proteobacteria (*Raoultella ornithinolytica* OKOH-1 and *Ensifer adhaerens* NWODO-2) and bacillus (*Bacillus* sp. NWODO-3, *Bacillus* sp. MABINYA-1, *Bacillus* sp. MABINYA-2 and *Bacillus* sp. FALADE-1), isolated from Tyhume river and Hogsback forest reserve in the Raymond Mhlaba Municipality, Eastern Cape, South Africa showed promising ligninolytic potentials as they were able to degrade some lignin monomers (guaiacol and veratryl alcohol) and decolourize selected lignin-mimicking dyes (Azure B, RBBR and CR). It is noteworthy that this is the first report of ligninolytic bacterial from Tyhume river and Hogsback in South Africa. The identified ligninolytic bacteria strains in this study fall within the classes of reported ligninolytic bacterial: actinomycetes, alpha-proteobacteria and gamma-proteobacteria (Bugg *et al.*, 2011). However, after the classification by Bugg and colleagues, recent reports had implicated some Bacillus species in lignin degradation

activities (Chang *et al.*, 2014; Zhu *et al.*, 2017). In other words, the classification of ligninolytic bacteria now includes *Bacillus* species. The findings from this study is consistent with previous related studies as Bao *et al.* (2015) reported the ligninolytic activity of *R. ornithinolytica* S12 isolated in China while Bandounas *et al.* (2011) reported the ligninolytic potential of *Bacillus* sp. LD003 isolated in Netherlands. Also, Chang *et al.* (2014) reported the lignin-degrading activity of *Bacillus* sp. CS-1 and *Bacillus* sp. CS-2 from forest soils in Japan while Zhu *et al.* (2017) reported the lignin degradation potential of *Bacillus ligniniphilus* L1 isolated from the South China Sea. Nevertheless, this seemed to be the first report on the ligninolytic potentials of Raoultella, Ensifer and Bacillus *species* in South Africa.

9.2.2. Optimization of environmental and nutritional conditions for peroxidase production

One of the major hindrances to the industrial application of peroxidases is the minute quantity of the enzyme produced. It is therefore imperative to optimize peroxidase production to meet its increasing market demand that may arise from the increase in industrial utility of peroxidase. More so, enhanced enzyme production is one of the important requirements for an effective biocatalytic process. Therefore, three (3) ligninolytic bacterial (*R. ornithinolytica* OKOH-1, *E. adhaerens* NWODO-2 and *Bacillus* sp. FALADE-1) which exhibited the most promising potential for peroxidase production (Table 3.4 and Table 4.3.) were optimized using the conventional approach which involved optimization of the bacteria environmental conditions (pH, temperature and agitation speed) as well as manipulating the nutritional conditions (carbon and nitrogen sources) of the fermentation medium for enhanced peroxidase production. Peroxidase production by *Bacillus* sp. FALADE-1 was optimal at pH 8 (Fig. 5.1), 30 ^{o}C (Fig. 5.2) and 150 rpm (Fig. 5.3) while that of *E. adhaerens* NWODO-2 was optimal at pH 7 (Fig. 6.1), 30 ^{o}C (Fig. 6.2) and 100 rpm (Fig. 6.3). For *R. ornithinolytica* OKOH-1, the optimum peroxidase production was observed at pH 5 (Fig. 7.1), 35 ^{o}C (Fig.7.2) and 150 rpm (Fig.7.3). These findings showed that optimal conditions for peroxidase production differ by bacterial species. This is corroborated by previous related studies that have documented various optimal conditions for peroxidase production by different bacterial species. Nour El-Dein *et al.* (2014) recorded optimum peroxidase production by *Streptomyces* sp. K37 at pH 7.5 and 40 ^{o}C while Musengi *et al.* (2014) observed optimum peroxidase production by *Streptomyces* sp. BSII#1 at pH 8 and 37 ^{o}C. On the other hand, Rekik *et al.* (2015) reported maximum peroxidase production by *Streptomyces griseosporeus* SN9 at pH 8,

45 ^{o}C and 180 rpm. Likewise, 180 rpm supported optimum peroxidase production by *Bacillus megaterium* (Patil, 2014). It is therefore clear that cultural environmental factors which influence bacterial growth play significant roles in peroxidase production (Jing and Wang, 2012; Rekik *et al.,* 2015) as previous studies had linked bacterial growth to constant secretion of extracellular enzymes in the culture medium (McCarthy, 1987; Musengi *et al.,* 2014).

Moreover, some lignin monomers have been reported to produce inductive effects on peroxidase production by bacteria (Musengi *et al.,* 2014; Rekik *et al.,* 2015). Therefore, this study evaluated the effects of selected lignin monomers (guaiacol, veratryl alcohol, vanillin, vanillic acid and ferulic acid) on peroxidase production by the studied ligninolytic bacteria. The findings showed that only guaiacol produced an inductive effect on peroxidase production by both *Bacillus* sp. FALADE-1 (Fig. 5.4) and *E. adhaerens* NWODO-2 (Fig. 6.4) while all the tested compounds induced peroxidase production in *R. ornithinolytica* OKOH-1 (Fig. 7.4). It is interesting to note that supplementation of the fermentation medium with guaiacol gave the highest peroxidase production in all the studied bacteria, hence guaiacol is the best inducer of peroxidase in the organisms. On the contrary, Musengi *et al.* (2014) reported veratryl alcohol as the best inducer of peroxidase in *Streptomyces* sp. strain BSII#1 while 2, 4-dichlorophenol (2, 4-DCP) produced the best inductive effect on peroxidase production by *Streptomyces griseosporeus* SN9 (Rekik *et al.*, 2015).

Furthermore, nitrogen source is another major factor that influence bacteria growth. Nonetheless, the nature of nitrogen source in the fermentation medium has produced different effects on production of lignin modifying enzymes by microbes. In fact, the effects of nitrogen on production of lignin modifying enzymes including peroxidase are not always consistent. In this study, peroxidase production by *Bacillus* sp. FALADE-1 and *E. adhaerens* NWODO-2 was enhanced by supplementing the fermentation medium with ammonium sulphate (Fig. 5.5, Fig. 6.5). However, supplementation of the medium with ammonium chloride gave the best peroxidase yield in *R. ornithinolytica* OKOH-1 (Fig. 7.5). It is evident from this study that, it is the sulphate and chloride ions of the ammonium salts that affected the peroxidase production by the respective organisms during nitrogen supplementation and not the ammonium. These findings are in agreement with previous related study by Prasher and Chauhan (2015), where it was reported that inorganic nitrogen sources supported maximum peroxidase production, whereas Mikiashvili *et al.* (2006)

gave a contradictory result which showed that inorganic nitrogen sources decreased production of peroxidase and other lignin modifying enzymes. Also, some authors had reported repression of peroxidase production by additional nitrogen (Kachlishvili *et al.*, 2005). The reasons for these discrepancies are not clear.

Under optimized conditions, *Bacillus* sp. FALADE-1 had a specific peroxidase productivity of 8.32 U mg^{-1} at 48 h (Fig. 5.6), *E. adhaerens* NWODO-2 exhibited a higher specific productivity of 12.76 U mg^{-1} also at 48 h (Fig. 6.6) while *R. ornithinolytica* OKOH-1 showed the highest peroxidase production with specific productivity of 16.48 U mg^{-1} at 72 h (Fig. 7.6). This finding suggests *R. ornithinolytica* OKOH-1 as the most efficient peroxidase producer in this study. Optimum peroxidase production by *Bacillus* sp. FALADE-1 and *E. adhaerens* NWODO-2 at 48 h augurs well for industrial production. More so, the short incubation period for optimum peroxidase production by all the tested bacteria is advantageous over fungi which are characterized by long incubation period (Robinson *et al.*, 2014; Prasher and Chauhan, 2015).

9.2.3. Molecular characterization of bacterial peroxidases

Peroxidases are a large group of enzymes with wide distribution across all forms of life including microbes. They are broadly classified into heme-containing and non-heme peroxidases. However, heme-peroxidases are more abundant in nature as over 73% of sequence data in the peroxidase database (PeroxiBase) encode heme-peroxidases (Zamocky and Obinger, 2010). Characterization of the gene encoding peroxidases is significant to gene cloning and over expression towards molecular optimization of peroxidase production. The genes encoding bacterial peroxidases can be identified using the N-terminal amino acid sequences and molecular weights of purified enzymes as well as the genomic data of the synthesizing microbes (Tamano, 2014). In this study, the sequence data generated from the PCR amplification of peroxidase genes in the peroxidase-producing bacteria and Sanger sequencing analysis, were used to characterize the different peroxidases. The nucleotide sequences of the peroxidase-encoding genes in the organisms were deposited in the GenBank of NCBI as RAOORO1, ENSADN2 and BAFPrx1 with MF370527, MF374336 and MF407314 as respective accession numbers.

Based on phylogenetic analysis and the use of bioinformatic software, peroxidases from *Bacillus* sp. FALADE-1 and *E. adhaerens* NWODO-2 were characterized as catalase-peroxidases with

estimated molecular weights of 11.445 kDa and 33.145 kDa respectively. More so, the deduced amino acid sequences of catalase-peroxidases from *Bacillus* sp. FALADE-1 and *E. adhaerens* NWODO-2 had isoeletric points of 7.01 and 11.47 respectively. Catalase-peroxidases are a unique class of heme-peroxidases with the ability to express both catalase and peroxidase activities. The presence of catalase-peroxidase genes in these two bacteria also indicates their potentials for production of a bifunctional enzyme and this might be the basis for their peroxidase activities as observed in this study. However, characterization of peroxidase gene from *R. ornithinolytica* OKOH-1 suggests it encodes a novel DyP-type peroxidase with an estimated molecular weight of 17.587 kDa and isoelectric point of 4.51. DyP-type peroxidases are novel peroxidases characterized by efficient decolourization of anthraquinone and azo dyes (Sugano *et al.,* 2007; Roberts *et al.,* 2011; Chen *et al.,* 2015). Molecular characterization of peroxidases in this study further confirms the ligninolytic potentials of the studied organisms as catalase-peroxidases and Dyp-type peroxidases are the major bacterial peroxidases implicated in lignin degradation (Brown *et al.,* 2011; de Gonzalo *et al.,* 2016).

9.2.4. Biochemical characterization of bacterial peroxidases

The increasing industrial applications of peroxidases have necessitated the search for new peroxidase with unique biochemical properties that would be of industrial significance. Hence, crude peroxidase from the most efficient producer in this study, *R. ornithinolytica* OKOH-1 was characterized using biochemical approaches such as substrate specificity, effect of pH and temperature on the peroxidase activity and stability as well as the effect of metal ions and possible inhibitors on the enzyme activity. Discussion of the findings from the biochemical characterization are presented in the succeeding section.

9.2.4.1. Substrate specificity

Peroxidase from *R. ornithinolytica* OKOH-1 (RaoPrx) had a wide substrate specificity as it was able to oxidize all the tested substrates: ABTS, veratryl alcohol, guaiacol and pyrogallol except 2, 6-dimethoxylphenol. However, the highest peroxidase activity was observed on pyrogallol with a K_m of 3.80 mmol L^{-1} and V_{max} of 4.65 µmol mL^{-1} min^{-1} (Fig. 8.1). This finding suggests that pyrogallol is the best substrate for RaoPrx. However, the best substrate for peroxidase from *Streptomyces griseosporeus* SN9 was 2, 4-dichlorophenol (Rekik *et al.,* 2015) while *Nonomuraea*

gerenzanensis peroxidase had the highest affinity for catechol (Casciello *et al.,* 2017). Detection of peroxidase activity on veratryl alcohol indicates a lignin peroxidase-like activity. More so, the ability of RaoPrx to oxidize both phenolic and non-phenolic compounds suggest its biotechnological potential for degradation of a wide range of xenobiotics in the environment.

9.2.4.2. Optimal pH and temperature

The optimum catalytic reaction of an enzyme is largely dependent on pH and temperature. Therefore, it is important to determine the pH and temperature that support an enzyme's optimum activity in a catalytic reaction. In this study, the optimal pH and temperature for RaoPrx activity were determined using a pH range 3.0 – 7.0 and a temperature range of 30 ^{o}C – 60 ^{o}C. RaoPrx activity was optimum at pH 6.0 (Fig. 8.2a) and 50 ^{o}C (Fig. 8.3a). These findings are comparable to previous studies. Fodil *et al.* (2011) reported an optimum pH range of 6.0 - 7.5 for a peroxidase from *Streptomyces* sp. AM2 while Olajuyigbe *et al.* (2015) recorded 50 ^{o}C as the optimum temperature for a crude peroxidase from *Actinomyces viscosus*. However, *Pseudomonas* sp. SUK 1 peroxidase had an optimum pH of 3 and temperature optimum of 40 ^{o}C (Kalyani *et al.,* 2011).

9.2.4.3. pH and thermal stability

Stability of an enzyme at an extreme pH and high temperature is desirable as these characterize the industrial applicability of such enzyme. As increase in temperature increases the rate of a catalytic reaction, temperature increase beyond the optimum temperature for a longer time may cause a denaturation of the enzyme. Hence, the interest in peroxidases that are able to withstand very high temperatures for a long period of time. This study found out that RaoPrx was stable over a pH range of 5.0 – 7.0 after 120 min of incubation. It was most stable at pH 6 with residual activity of over 50 % after 120 min (Fig. 8.2b). It is interesting to note that RaoPrx was very stable at higher temperatures (50 ^{o}C -70 ^{o}C) as it retained over 70 % of its original activity after 120 min incubation time (Fig. 8.3b). Moreover, the enzyme stability at 50 ^{o}C was outstanding as it retained about 93 % of its original activity after the incubation of period of 120 min. The stability of RaoPrx within a short pH range is consistent with previous studies (Fodil *et al.,* 2012; Olajuyigbe *et al.,* 2015; Casciello *et al.,* 2017). It is worthy of note that the thermal stability of RaoPrx is higher than most previously reported microbial peroxidases as peroxidase from *Rhizoctonia* sp. SYBC-M3 was only stable at temperatures below 50 ^{o}C (Cai *et al.,* 2010) while peroxidases from two

actinomyces species completely lost their activities at 70 ^{o}C after 150 min (Olajuyigbe *et al.*, 2015). Even, peroxidase from one of the most efficient white-rot fungi, *Phanerochaete chrysosporium* completely lost its activity at 65 ^{o}C after 60 min (Urek and Pazarlioglu, 2004). The high thermostability exhibited by RaoPrx suggests its potential for industrial applications.

9.2.4.4. Effect of metal ions and inhibitors

Determining the effect of different metal ions is significant to the industrial applicability of any enzyme as the effect elicited by some common inhibitors may give an insight about the nature of the enzyme being investigated. In this study, the activity of RaoPrx was enhanced by Ag^{+}, $Cu^{2+,}$ Zn^{2+} and Fe^{2+} while Ca^{2+}, Mg^{2+}, Ba^{2+}, Al^{3+} and NaN_3 partially inhibited the enzyme activity. However, RaoPrx activity was completely inhibited by Co^{2+} and EDTA (Table 8.3). To a large extent, the effects of the metal ions are dose-dependent as higher concentration produced a more significant effect on the enzyme activity. Generally, findings on the effects of metal ions on enzyme activities seemed not to be consistent as previous studies had reported different effects for the same metal ions on peroxidase activity. For instance, Fodil *et al.* (2012) reported that Ca^{2+} and Cu^{2+} increased the activity of peroxidase from *Streptomyces* sp. AH4 while $Zn^{2+,}$ Mg^{2+} and Co^{2+} were reported to have inhibitory effects on the enzyme activity. On the other hand, the activity of peroxidase from *Streptomyces griseosporeus* SN9 was moderately inhibited by Ba^{2+} and Co^{2+} while Cu^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+} did not elicit significant effect on the enzyme activity (Rekik *et al.*, 2015) whereas Olajuyigbe *et al.* (2015) reported that the activity of peroxidase from *Actinomyces viscosus* was enhanced by Ca^{2+} and Fe^{2+} while the enzyme activity was inhibited by Cu^{2+} and Mg^{2+}. As observed in this study, the authors also reported the inhibitory effect of Ca^{2+} on the activity of peroxidase from *Actinomyces israelii* which is rather rare. It is therefore evident that different metal ions elicit different effects on enzyme activity which are likely dependent on the charges on the amino acid residues in the active sites of the enzyme and the conformational change that occurs as a result of the interaction of the metal ions with the amino acid residues in the enzyme catalytic site (Olajuyigbe and Ogunyewo, 2016).

Furthermore, the inhibition of RaoPrx activity by EDTA, which is consistent with previous related studies (Asgher *et al.*, 2012; Praveen *et al.*, 2012; Olajuyigbe *et al.*, 2015), is perhaps attributed to the metal chelating activity of EDTA which rendered the cofactors of peroxidase unavailable for

catalytic reaction. This is suggestive of the likely dependence of RaoPrx on a heme component for its catalytic reaction (Fodil *et al.,* 2011). Likewise, complete inhibition of RaoPrx activity by Co^{2+} is probably due to the ability of cobalt ions to form complexes or it might be that the ion reacted with hydrogen peroxide (Atabey *et al.,* 1996), which was supposed to initiate the catalytic reaction, thereby making it unavailable to activate the enzyme as appropriate.

9.2.5. Biotechnological potentials of peroxidases in textile dye remediation and development of cosmetic agents

Textile industries are major consumers of dyes, which are toxic substances with potential negative impacts on environmental and human health as significant amount of dyes are released into the environment in the form of textile effluent from the industries (Yanto *et al.,* 2014). The presence of dyes in the environment has been a major concern as this may constitute an environmental nuisance probably due to the recalcitrance of textile dyes and the fact that most of these dyes and their degradation products might be toxic (Singh *et al.,* 2015). Consequently, in our recent review (Falade *et al.,* 2017), we emphasized the imperativeness of developing effective and efficient strategy for dye removal as most of the current approaches (Chemical methods) used in dye decolourization and treatments are limited by high cost and secondary pollution problems (Parshetti *et al.,* 2012). Nevertheless, biological methods of dye decolourization which involve the use of microorganisms and biomolecules including enzymes have been suggested (Falade *et al.,* 2017). In this study, crude peroxidase from *R. ornithinolytica* OKOH-1 was evaluated for decolourization of selected azo (Congo red) and anthraquinone (Remazol Brilliant Blue R and Reactive blue 4) dyes as well as synthetic melanin. The study showed that RaoPrx exhibited decolourizing activity on all the dyes investigated. However, the enzyme displayed a remarkable dye-decolourizing activity on congo red (65.03%) and melanin (47.96 %) within 30 min of incubation (Fig. 8.7). The effectiveness of RaoPrx on congo red within a short time indicates that azo dyes are probably more susceptible to decolourization by the enzyme than anthraquinone dyes as dye degradation can be attributed to susceptibility of the arene substituents to hydrozylation (Goszczynski *et al.,* 1994). Congo red has its arene substituents attached at the *ortho* position while reactive blue 4 and remazol brilliant blue r have their aromatic substituents attached at the *para* positions (Fig. 3.2). It is therefore suffice to suggest that dyes with *ortho* arene substituents might be more susceptible to decolourization than the *para* arene substituent counterparts. This claim is

in agreement with Murugesan *et al.* (2006) who attributed differences in the rate of dye decolourization to structural variations of the dyes. This finding suggests the biotechnological potential of crude peroxidase from *R. ornithinolytica* OKOH-1 for textile dye remediation. My finding agrees with previous studies which had also reported the potential of bacterial peroxidases for dye decolourization (Kalyani *et al.* 2011; Rekik *et al.*, 2015).

Furthermore, the ability of RaoPrx to decolourize a synthetic melanin, is an indication of its potential as a promising alternative to hydroquinone in the development of skin-lightening agents. Previous studies had also corroborated the potential of peroxidases for effective decolourization of synthetic melanin (Woo *et al.*, 2004; Nagasaki *et al.*, 2008).

9.3. Conclusions and future prospects

This study isolated novel ligninolytic bacterial species with promising peroxidase production potentials that could be exploited for biotechnological applications. The bacteria were identified as *Raoultella ornithinolytica* OKOH-1 (KX640917), *Ensifer adhaerens* NWODO-2 (KX640918) and *Bacillus* sp. FALADE-1 (KX640922) belonging to gamma-proteobacteria, alpha-proteobacteria and bacilli respectively. Upon optimization, the peroxidase production yield by the three organisms, increased by 3.40-fold, 1.09-fold and 2.22-fold respectively. The ability of *R. ornithinolytica* OKOH-1 and *E. adhaerens* NWODO-2 to utilize sawdust as the best substrate for enhanced peroxidase production under solid state fermentation is notable and significant as this would reduce the high cost of enzyme production which could be an impediment to large scale production of peroxidase by the bacteria.

It is interesting to note that this study was able to detect and characterize the genes encoding peroxidase production in the three producers. Through molecular approach, peroxidase from *R. ornithinolytica* OKOH-1 was characterized as a DyP-type peroxidase while the genes detected in both *E. adhaerens* and *Bacillus* sp. FALADE-1 encode catalase-peroxidases. The detection of the genes encoding peroxidases in the studied bacteria could further be exploited for molecular optimization of peroxidase production through gene cloning and over expression which could be an excellent tool towards industrial and large-scale enzyme production. I believe future research efforts should therefore be channeled in this direction.

The unique biochemical characteristics such as oxidation of a wide range of substrates and high thermostability displayed by the peroxidase from *R. ornithinolytica* OKOH-1 as well as its excellent dye decolourization potential augur well for its industrial applicability in the future. Nevertheless, optimization of dye decolourization process by peroxidase from *R. ornithinolytica* OKOH-1 is imperative and should be considered for further studies.

The potential of the identified bacteria for lignin degradation has been established in this study. However, further study should look into elucidating the mechanisms of action of lignin degradation and pathways employed by the organisms using molecular approaches such as proteomics, which may also include characterization of the genes implicated in ligninolytic activity. As well, the ligninolytic potential of these bacteria makes them promising candidates for biodegradation of a wide range of phenolic and non-phenolic compounds in the environment.

Finally, this study has identified *R. ornithinolytica* OKOH-1 as the most promising candidate for biotechnological applications as members of this genus have been implicated in a number of biotechnological processes including production of pullulanase, polysaccharide-protein complex, and tripeptide-complex. As well, they have also been implicated in the production of organic acids such as 2,3-Butanediol (2, 3 BD) as an alternative to the petroleum-based 2, 3 BD production. More so, the detection of multicopper oxidase gene in *R. ornithinolytica* OKOH-1 in the course of this study suggests its potential for laccase production. It is therefore recommended that the biotechnological potential of *Rornithinolytica* OKOH-1 should be further explored for production of other biomolecules of economic importance. In order to fully maximize the potential of this novel strain, its whole genome should be sequenced in the future.

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APPENDIX

Appendix A

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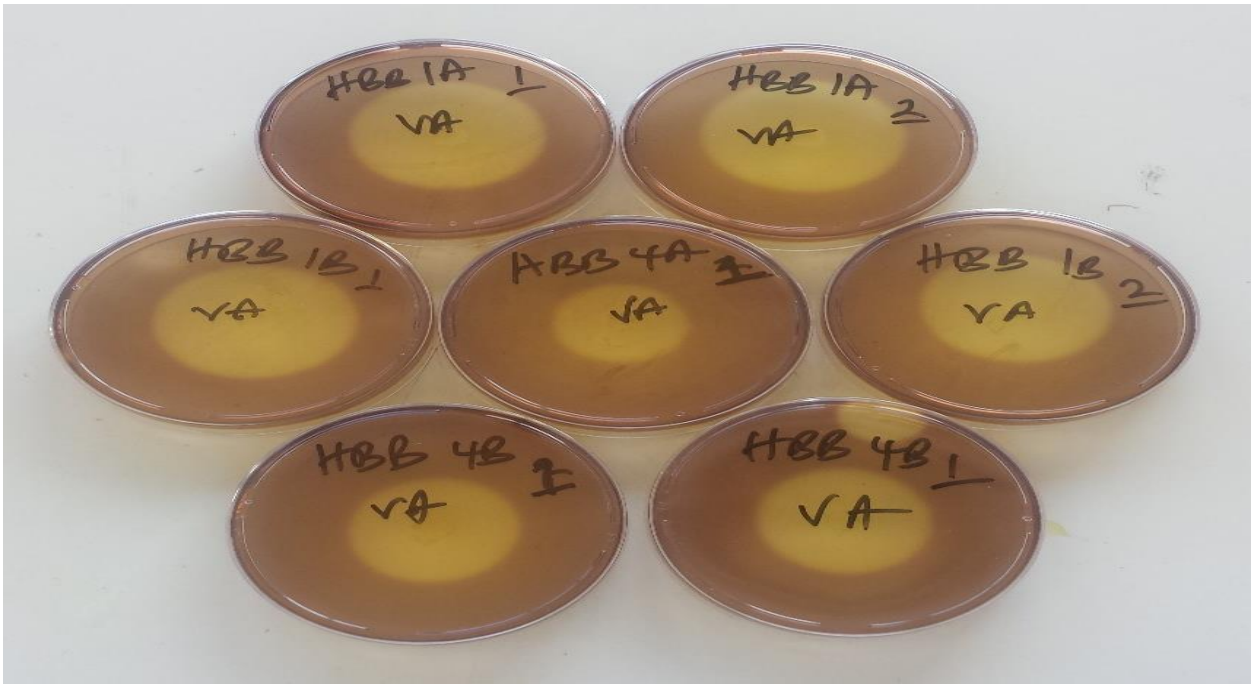
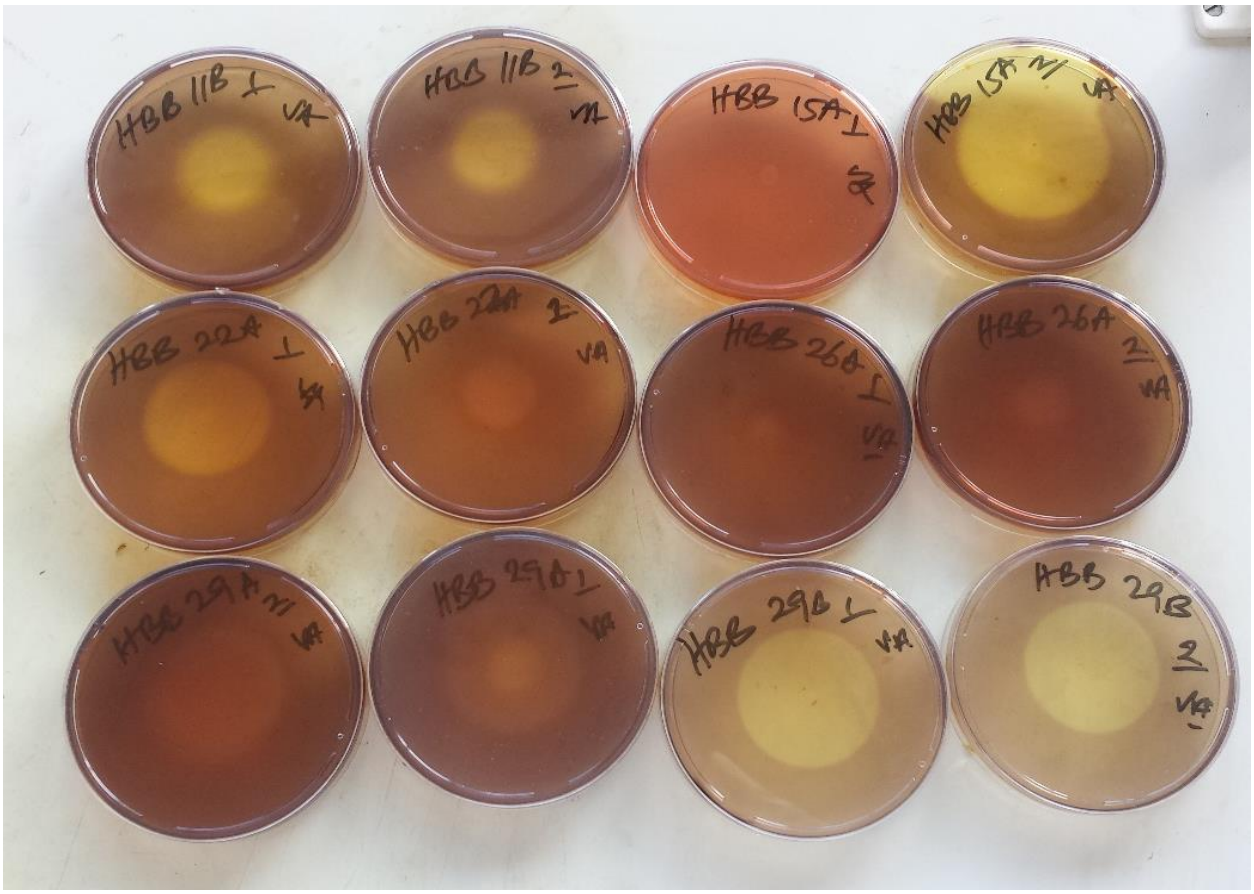
Appendix B

Plate screening of bacterial isolates for degradation of lignin model compounds

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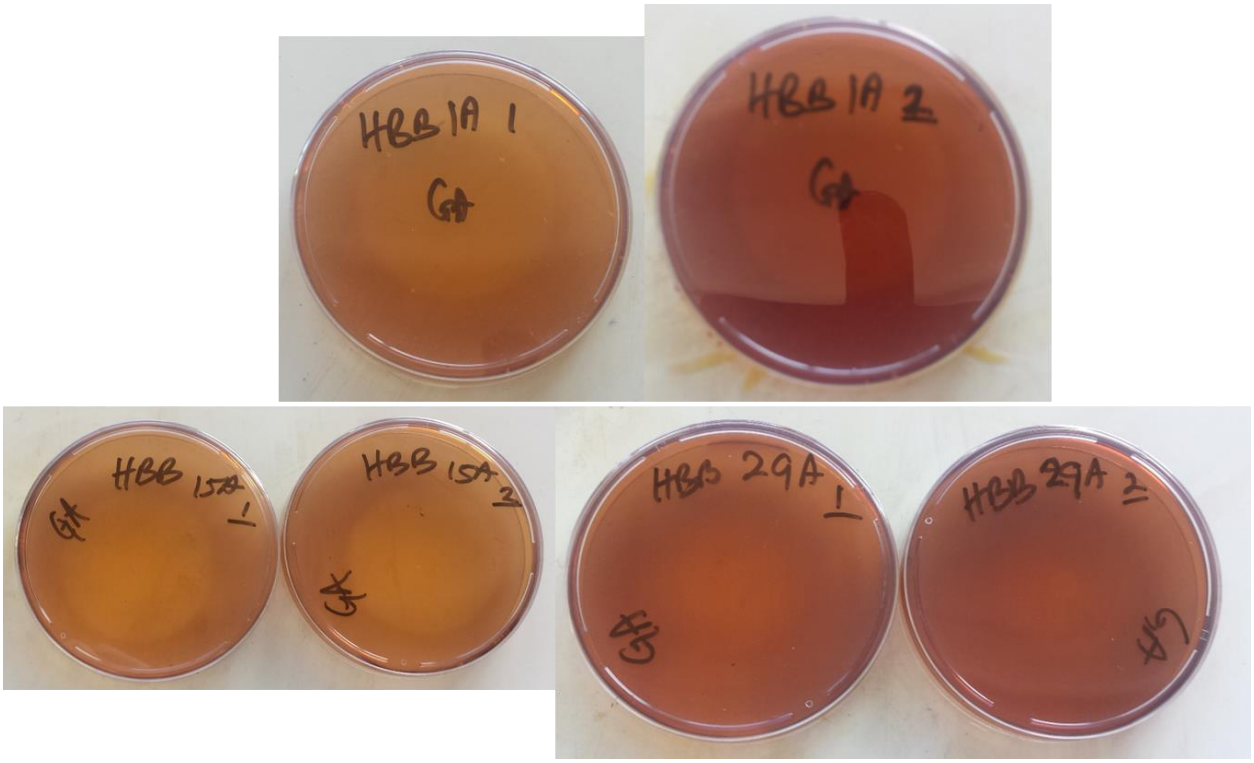
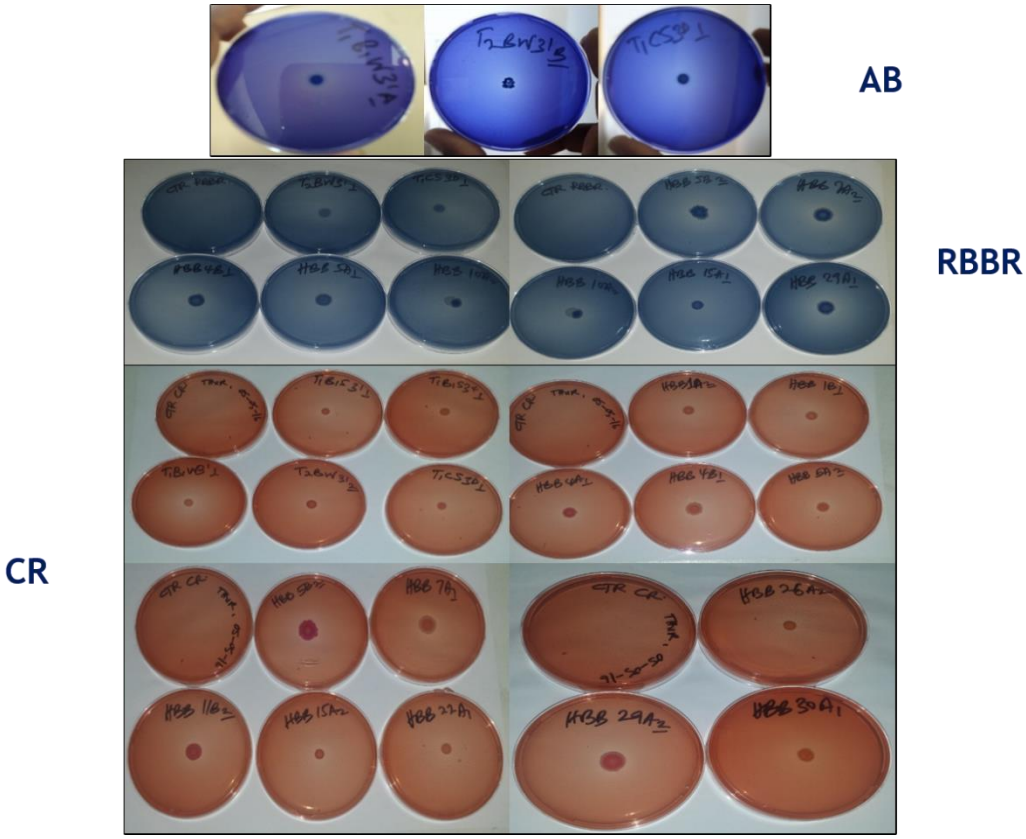
HBB 11B 2 VA
HBB 15A 1 VA
HBB 15A 2 VA
HBB 22A 1 VA
HBB 22A 2 VA
HBB 26B 1 VA
HBB 26A 2 VA
HBB 29A 2 VA
HBB 29B 1 VA
HBB 29B 1 VA
HBB 29B 2 VA


HBB 1A 1
GA
HBB 1A 2
GA
HBB 15A 1
GA
HBB 15A 2
GA
HBB 29A 1
GA
HBB 29A 2
GA

Appendix C

Plate screening of bacterial isolates for dye decolourization



AB: Azure B, CR: Congo Red, RBBR: Remazol Brilliant Blue R

Appendix D

Calculation of peroxidase activity

$$\text{U/mL} = \frac{\left(\frac{\Delta A}{\Delta t}\right) x\ Vt}{\in x\ Ve}$$

Where:

$\left(\frac{\Delta A}{\Delta t}\right)$ = Slope of plot of absorbance @ 420 nm against time in min

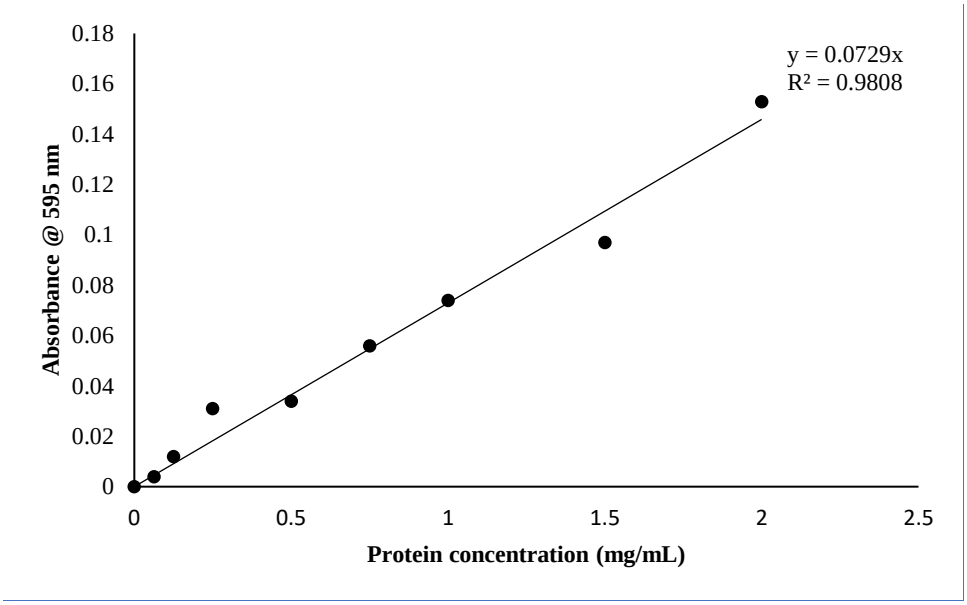
Vt = Total reaction volume (mL)

$\in$ = Extinction coefficient

Ve = Enzyme volume (mL)

Appendix E

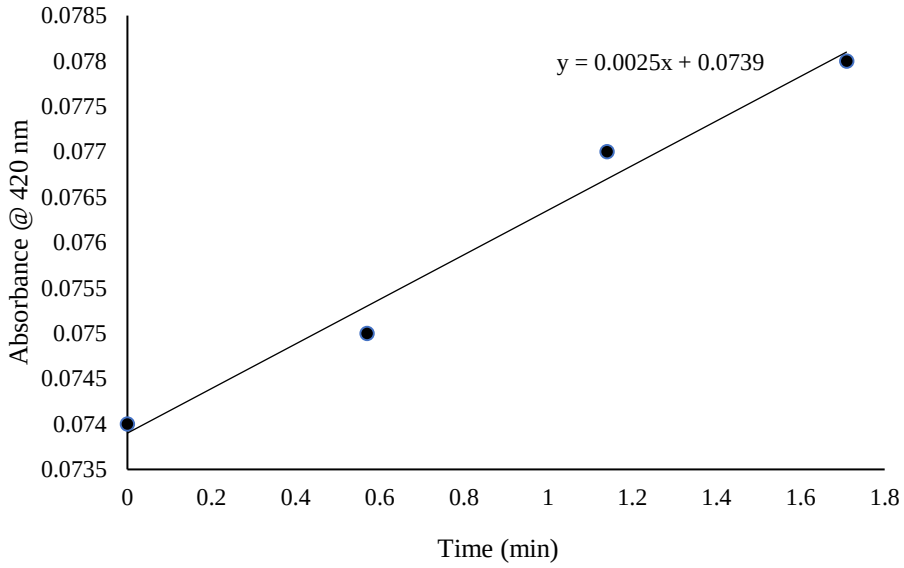
Standard curve of protein estimation



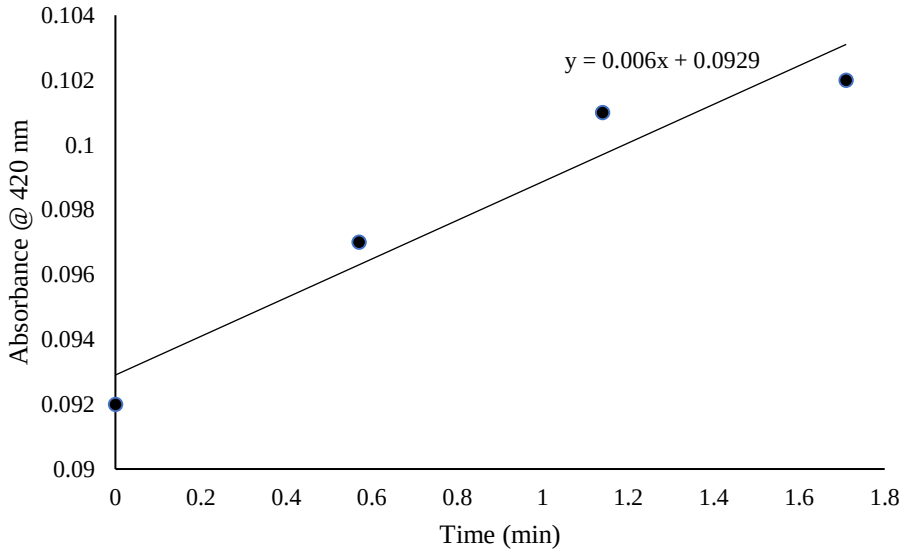
Appendix F

Selected kinetics graphs used for biochemical characterization of *Raoultella ornithinolytica* peroxidase (RaoPrx)

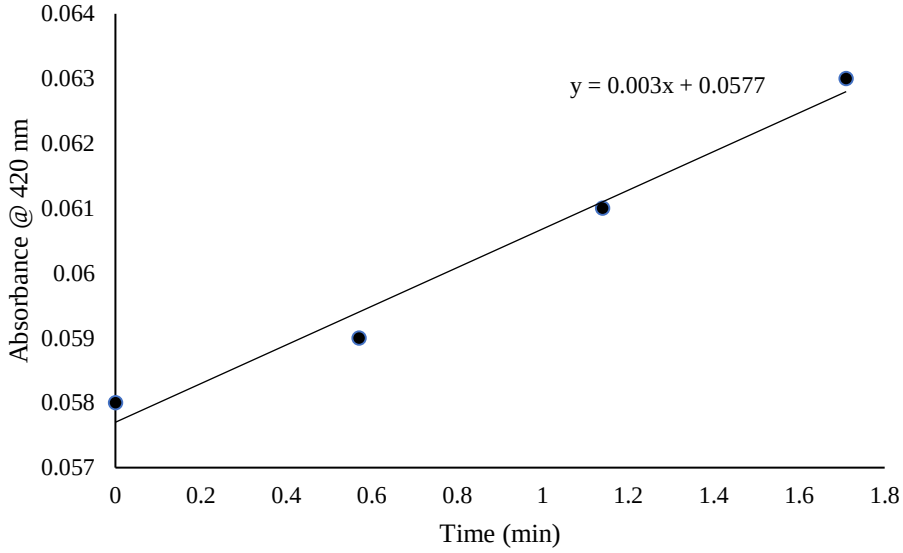
pH 5



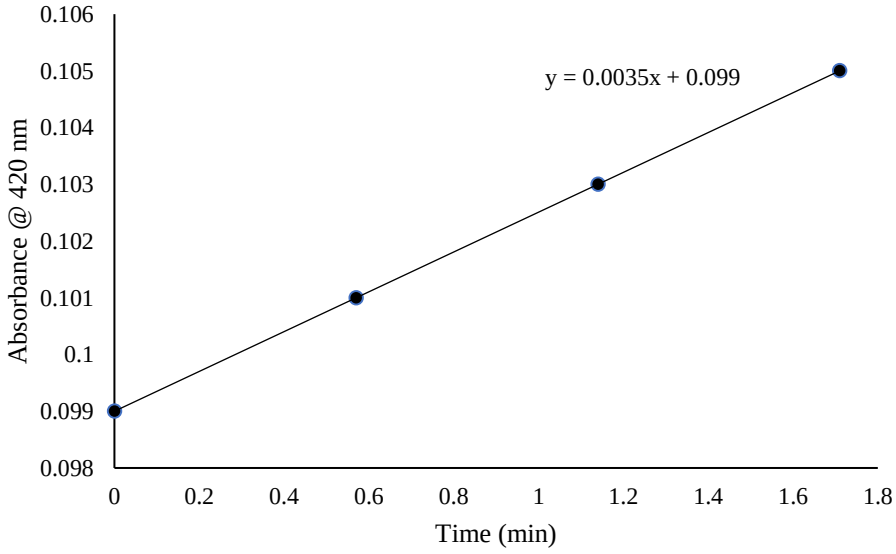
pH 6



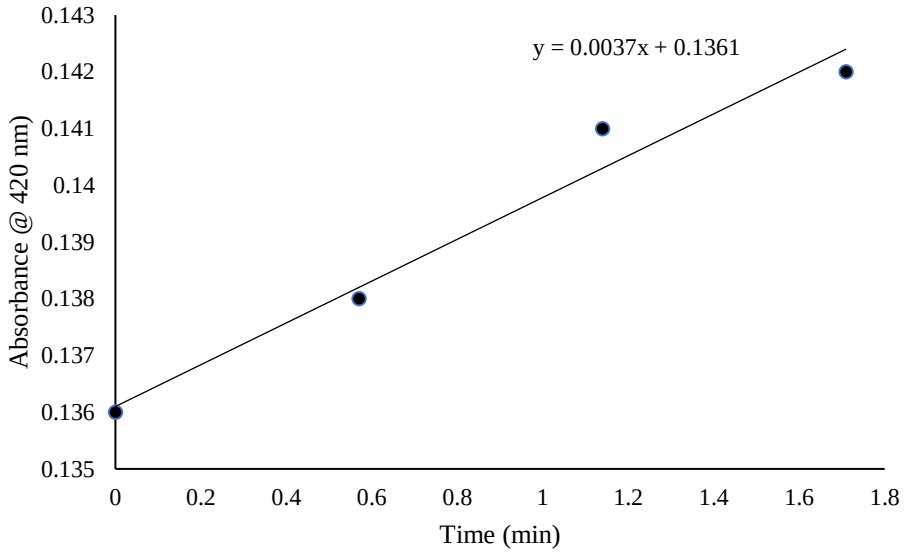
pH 7


y = 0.003x + 0.0577
Absorbance @ 420 nm
0.064
0.063
0.062
0.061
0.06
0.059
0.058
0.057
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Time (min)

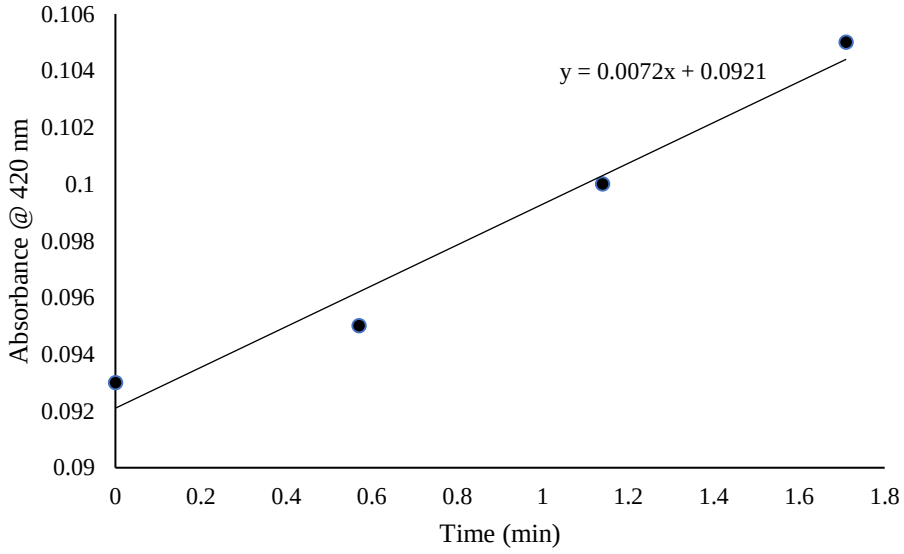
30 ºC


y = 0.0035x + 0.099
Absorbance @ 420 nm
0.106
0.105
0.104
0.103
0.102
0.101
0.1
0.099
0.098
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Time (min)

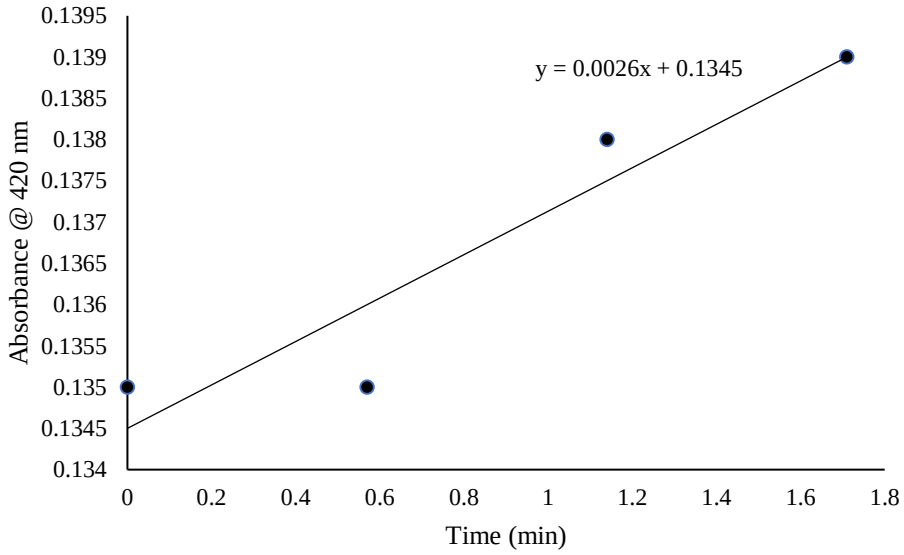
40 ºC


y = 0.0037x + 0.1361
Absorbance @ 420 nm)
0.143
0.142
0.141
0.14
0.139
0.138
0.137
0.136
0.135
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Time (min)

50 ºC


y = 0.0072x + 0.0921
Absorbance @ 420 nm
0.106
0.104
0.102
0.1
0.098
0.096
0.094
0.092
0.09
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Time (min)

60 ºC


y = 0.0026x + 0.1345
Absorbance @ 420 nm
0.1395
0.139
0.1385
0.138
0.1375
0.137
0.1365
0.136
0.1355
0.135
0.1345
0.134
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
Time (min)