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The degradation of the endocrine disrupting chemical, bisphenol-A: a comparative study between fungal and bacterial laccases

M.Sc Biotechnology

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A. ABSTRACT

The degradation of endocrine disrupting chemicals (EDCs) is a topic of high importance and one that research efforts are continually being focused on. These harmful chemicals are known to cause adverse health effects in humans and animals. In particular, bisphenol-A (BPA), a high volume chemical which is mainly used in the manufacturing of polycarbonate plastics and epoxy resins have been shown to be implicated in the development of a variety of health problems. In this study, the ability of two fungal laccases [*Trametes versicolor* (TvL) and *Trametes pubescens* (TpL)], and two bacterial laccases [*Streptomyces coelicolor* (SLAC), and a mutant of SLAC (SLAC-VN)] to degrade or remove BPA from solution was investigated. The commercial preparation of TvL was used for the purposes of this study, while TpL was produced from the native strain. *T. pubescens* was cultured in shake-flasks, the supernatant harvested and subjected to ammonium sulphate precipitation. SLAC and SLAC-VN were produced from recombinant strains using a standard protocol and the enzymes purified by size-exclusion chromatography. The presence of the laccases were confirmed by the 2,6-dimethoxyphenol assay and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The removal or degradation of BPA from solution was determined for the free enzymes, as well as the enzymes in immobilised form. For immobilisation, the enzymes were encapsulated in sodium alginate beads and cross-linked to form cross-linked enzyme aggregates (CLEAs).

High levels of BPA removal was exhibited by the fungal laccase, TpL (100% removal) and the bacterial mutant laccase, SLAC-VN (96%) in their free form. When all four

laccases were encapsulated in sodium alginate beads, a number of changes to the characteristics of the enzymes were observed. Overall, the level of BPA removal was reduced for all enzymes as when compared to the free laccases, while SLAC-VN removed more BPA than either of the fungal laccases (59% for SLAC-VN versus 57% TvL and 54% for TpL). The encapsulation of the laccases in alginate beads also led to changes in the optimal temperature for BPA removal, with all encapsulated laccase being able to remove BPA optimally at 40°C. The immobilisation of the laccases in CLEA form had the most significant effect on the BPA removal ability of the laccases. The pH range for both fungal laccases was extended beyond the acidic range [for TpL, optimal removal occurred at pH 8.5 compared to pH 4.5 (free) and pH 6.0 (encapsulated)]. Most remarkable, however, was that the formation of CLEAs greatly enhanced the BPA removal ability of SLAC (60% removal compared to 25% when encapsulated).

Furthermore, the effect of four known laccase mediators, two synthetic (1-HBT and TEMPO) and two natural (ferulic acid and vanillin), on the ability of the laccases to remove BPA was determined. The effect of the mediators on the ability of the laccases to remove BPA was mostly negligible, with the exception of SLAC, where a 6-fold increase in BPA removal was observed in the presence of the two natural mediators, ferulic acid and vanillin (7% with no mediator vs 28% in the presence of mediator). This suggests that in the presence of redox mediators, complete removal of BPA may be observed when CLEAs produced from the bacterial laccases are used.

LC-MS analysis of putative metabolites determined that the removal of BPA was mainly as a result of an oligomerisation process. BPA was oligomerised into dimers,

trimers and tetramers, and further degradation of the BPA oligomers was observed through the presence of breakdown intermediates, phenol and 4-isopropenylphenol. As part of a future study, the toxicity of these putative metabolites will be determined through the use of the Yeast Oestrogen Assay.

In this study, our results showed that the bacterial laccases were able to perform on a comparable level to the fungal laccase and demonstrated that, with further optimisation, these enzymes have the potential for application in industrial processes.

B. DECLARATION

I declare that "*The degradation of the endocrine disrupting chemical, bisphenol-A: a comparative study between fungal and bacterial laccases"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged by complete references.

Full name: Alaric Prins **Date:** 15 June 2015

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Signature

C. ACKNOWLEDGEMENTS

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- To my wonderful nephew and niece, who often drive me insane, but for whom I wouldn't trade anything in the world.
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E. LIST OF ABBREVIATIONS

Abbreviation	
1-HBT	1-hydroxybenzotriazole
2,6-DMP	2,6-dimethoxyphenol
3-HAA	3-hydroxyanthranilic acid
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AC	Activated carbon
AP	Alkylphenol
AR	Androgen receptors
BPA	Bisphenol A
BSA	Bovine serum albumin
CLEA	Cross-linked Enzyme Aggregates
CLEC	Cross-linked Enzyme Crystals
DME	1,2-dimethoxyethane
EDC	Endocrine disrupting chemical
EPA	Environmental Protection Agency
ER	Oestrogen receptors
FPLC	Fast performance liquid chromatography
GAC	Granular Activated Carbon
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
IVF	*In vitro* fertilisation
kDa	Kilodalton
LC-MS	Liquid chromatography-Mass spectrometry
LMS	Laccase Mediator System
NOAEL	No observed Adverse Effects Level
NP	Nonylphenol
OP	Octylphenol
PAC	Powdered Activated Carbon
PAN	Polyacrylonitrile
PCB	Polychlorinated dibenzophenols and dibenzofurans
PCOS	Polycystic Ovarian Syndrome
PR	Progesterone receptors
RCBA	Recombinant Yeast Cell Bioassay
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SLAC	Small laccase from *Streptomyces coelicolor*
SLAC-VN	SLAC mutant, SLAC-V290N
TEMPO	2,2,6,6-tetramethylpiperidin-1-yl)oxy
TpL	*Trametes pubescens* laccase
TR	Thyroid receptors
TvL	*Trametes versicolor* laccase
UV	Ultraviolet
VLA	Violuric acid

F. LIST OF FIGURES

G. LIST OF TABLES

H. RESEARCH OUTPUTS

- Prins, A., Kleinsmidt, L., Khan, N., Kirby, B., Kudanga, T., Vollmer, J., Pleiss, J., Burton, S. and Le Roes-Hill, M. (2015) The effect of mutations near the T1 copper site on the biochemical characteristics of the small laccase from *Streptomyces coelicolor* A3(2). *Enzyme and Microbial Technology,* 68: 23-32
- 7th International Congress on Biocatalysis, 31 August-4 September 2014 (Hamburg University of Technology, Germany) Small laccase (SLAC) and SLAC mutants as biocatalysts for the synthesis of potent antioxidants. **T Kudanga, L Kleinsmidt, S Mbulawa, A Prins, J Pleiss, M Le Roes-Hill** (poster presentation).
- Enzyme Engineering XXII: Emerging Topics in Enzyme Engineering, 22-26 September 2013 (Toyama, Japan) The effects of a mutation, M298F, on the biochemical characteristics of the small laccase from *Streptomyces coelicolor* A3(2). **A Prins, N Khan, B Kirby, J Vollmer, J Pleiss, S Burton, M Le Roes-Hill** (poster presentation).
- South African Society for Microbiology (SASM) 2013 conference, 24-27 November, Forever Resorts Warmbaths, Bela-Bela. The effects of a mutation, M298F, on the biochemical characteristics of the small laccase from *Streptomyces coelicolor*. **A Prins, N Khan, B Kirby, J Vollmer, J Pleiss, S Burton, M Le Roes-Hill** (poster presentation).

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

In an era where sustainability is becoming increasingly important, research is gradually geared towards the challenge of ensuring that there is a safe and sustainable supply of drinking water, with a particular focus on the development of technologies for the regeneration of potable water from wastewater. A number of factors have played a contributing role in reducing the quality of surface waters, including but not limited to, population growth, urbanisation, industrial development, and the associated changes in agricultural and other land-use practices (Levine and Asano, 2004; Falconer *et al.,* 2006).

1.2 Endocrine Disrupting Chemicals

Amongst a number of environmental pollutants, one group of compounds, the endocrine disrupting chemicals (EDCs), have been at the centre of an increasing number of research efforts over recent years. According to the United States Environmental Protection Agency (EPA), an endocrine disrupter is defined as "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action and elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behaviour" (U.S. EPA, 1997).

"Endocrine disruption" can be broadly interpreted to include both agonists and antagonists of steroid hormone receptors, the modulation of enzymes that are involved in the synthesis or metabolism of hormones, and the disruption through alteration of both molecular and biochemical events, including pathways that are involved in cellular signalling or gene expression (Figure 1.1) (Kudlak and Namiesnik, 2008; Phillips and Foster, 2008). The typical EDC targets are nuclear receptors such as oestrogen receptors (ER), androgen receptors (AR), progesterone receptors (PR) and thyroid receptors (TR) (Vuorinen *et al.,* 2013).

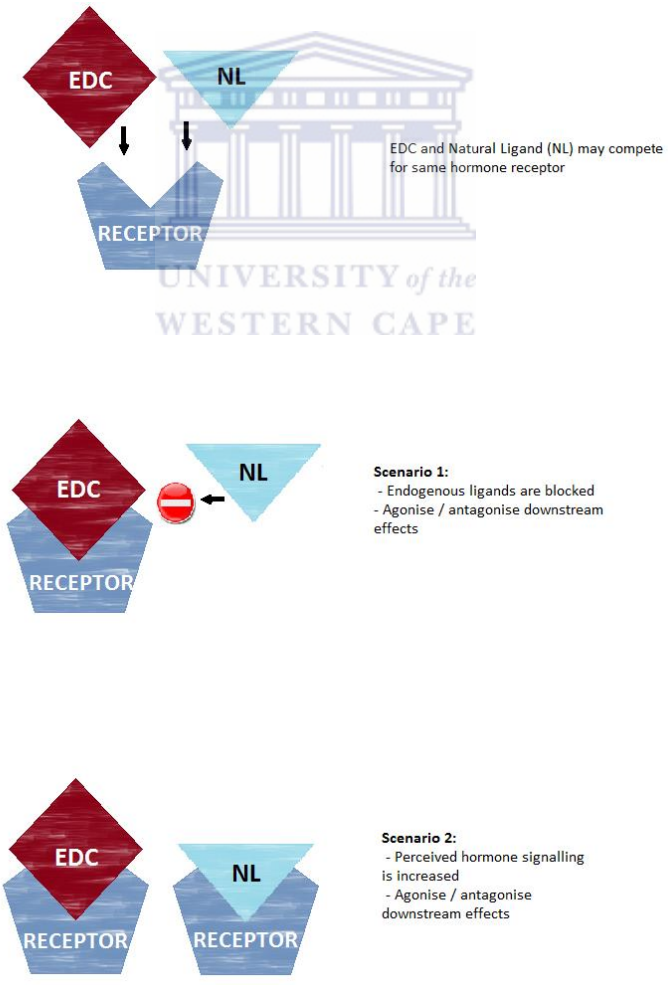

Figure 1.1: Schematic representation of the interaction between a hormone receptor and endogenous ligand/EDCs (modified from Rogers *et al.,* 2013)

Environmental pollutants considered to be EDCs are often classified according to one of three classes (Table 1.1). These classes are industrial chemicals, natural hormones and pesticides, and may originate from a variety of sources, both synthetic and natural, and typically includes steroid sex hormones, personal care products, pharmaceutical residues, antiseptics, brominated flame retardants, alkylphenol ethoxylates, and gasoline additives (Kudlak and Namiesnik, 2008; Bicchi *et al.,* 2009).

Table 1.1: Classification of environmental pollutants considered to be EDCs (adapted from Falconer *et al.,* 2006; Kudlak and Namiesnik, 2008)

Class	Description
Class I: Industrial chemicals	Plasticizers (phthalates applied in polymer industries) Alkylphenols Bisphenol-A Polychlorinated dibenzophenols and dibenzofuranes (PCBs) Dioxins Brominated flame retardants Parabens (cosmetics) Butylated hydroxyanisoles (BHAs) Surfactants and detergents Natural and synthetic musk
Class II: Natural hormones	Phytoestrogens Female hormones [oestrone (E1); 17β-estradiol (E2)] Mycotoxins
Class III: Pesticides	DDT Amitraz Carbofuran Trichlorfan Atrazine Benomyl, and others

1.3 Bisphenol-A

Among the aforementioned EDCs (Table 1.1), the industrial chemical bisphenol-A (BPA), is perhaps the most widely known. BPA [2,2-bis-(4-hydroxyphenyl)propane, CAS# 80-05-07] is an industrial compound that is synthesised by the condensation of two phenol groups and one acetone molecule (Figure 1.2) (Geens *et al.,* 2012). More

than 2.7 billion kilograms of BPA is produced worldwide, per annum (Mok-Lin *et al.,* 2009). BPA is mainly used in the manufacturing of polycarbonate plastics and epoxy resins, which has a wide range of applications. These include composites and sealants in the dental industry (Joskow *et al.,* 2006; Fleisch *et al.,* 2010), reusable plastic bottles, baby feeding bottles, microwave containers, internal coatings for food and beverage cans, sunglasses, building materials, CD-ROMs and medical devices (Geens *et al.,* 2012). BPA has been detected in a variety of environmental samples, including water, sewage leachates, indoor and outdoor air samples and dust (Vandenberg *et al.,* 2007). Public awareness of the implications of BPA exposure is increasing. As recently as April 2014, a popular South African news website posted an article on the dangers of BPA exposure in which the article stated that many plastic producers are now circumventing health regulations prohibiting the use of BPA in bottles by incorporating alternative chemicals that also exhibit oestrogenic activity (Wilson-Späth, 2014).

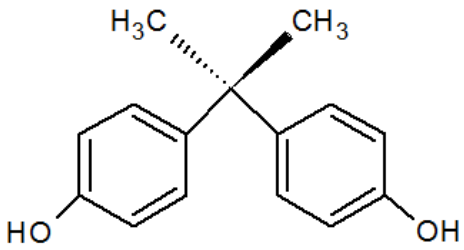

Figure 1.2: The chemical structure of BPA

1.4 Human Exposure to BPA

Studies have shown that incomplete polymerization of BPA during production processes and repeated exposure to heat and/or acidic/basic conditions (even in the case of completely polymerized BPA) may promote the leaching of BPA from plastics and coatings into the surrounding environment (Vandenberg *et al.,* 2010).

Human exposure to BPA is typically caused by the hydrolysis of polycarbonate plastics and epoxy resins, causing low concentrations of BPA to leach into foods and liquids (Crain *et al.,* 2007). The use of products that contain polycarbonate plastics and epoxy resins has increased, and has concomitantly led to the widespread exposure of the general population to BPA (Mok-Lin *et al.,* 2009). Biomonitoring studies have been performed in several countries. A comparative study of the daily BPA intake among United States (U.S.), Korean and Japanese populations, estimated the maximum daily intake of BPA per kilogram of body weight, to be approximately 0.23 µg/kg/day based on urinary BPA concentrations (Yang *et al.,* 2006). BPA has been measured in human tissues and fluid in many developed countries and a general consensus has been reached that BPA can be detected in most individuals in these countries (Vandenberg *et al.,* 2007). In a National Health and Nutrition Examination Survey (NHANES) held between 2003-2004 in the U.S., the total urinary concentration of BPA was measured among 2 517 participants above the age of six. BPA, in both free and conjugated form, was detected in the urine of 92.6% of the participants (Calafat *et al.,* 2008). In a total of 84 women undergoing *in vitro* fertilization (IVF), urinary BPA concentrations measured in a range of < 0.4 – 25.5 µg/L (Mok-Lin *et al.,* 2009).

Since the 1930s, BPA has been known to cause adverse effects on the endocrine system (Dodds and Lawson, 1936). These adverse effects are attributed to the ability of BPA to act as an oestrogen agonist and to promote the effect of endogenous 17β-estradiol (Crain *et al.,* 2007). Research on the kinetics of BPA binding to oestrogen receptors (ER), amongst other hormone receptors, has determined that BPA is able to

bind to both ERα and ERβ, with an almost 10-fold higher affinity to ERβ (Pennie *et al.,* 1998).

A “low dose” of BPA at 50 mg/kg/day was defined by the National Institute of Environmental Health Sciences (NIEHS) Low Dose Peer Review as doses below the accepted “no observed adverse effect level” (NOAEL) (Kortenkamp *et al.,* 2007). However, many studies have indicated that the effects of BPA exposure may be observed in animals exposed to < 50 mg/kg/day (Vandenberg *et al.,* 2010).

1.5 Toxicological effects of BPA in mammals

Exposure to BPA has been associated with a variety of adverse health effects (Table 1.2), which includes reproductive disorders, the formation of cancers and complications in the developmental stages of life (Lee *et al.,* 2013). Exposure to endocrine disruptors has different effects, which is dependent on the developmental stage of the exposed animal. The most significant effect was observed with exposure during organ development (starting at prenatal development and continuing until puberty) as exposure at this stage may result in irreversible changes (Richter *et al.,* 2007). Due to the limited amount of published work on the effects of exposure in humans, the majority of the research is based on animal models.

Table 1.2: Diseases that may arise from exposure to EDCs during development stages (adapted from Schug *et al.,* 2011)

System affected	Physiological effect	Chemicals
Reproductive/endocrine	Cancers – prostate/breast	BPA
	Infertility	Oestrogens, pesticides
	Diabetes/metabolic syndrome	BPA
	Early puberty	BPA, oestrogens
	Obesity	BPA, pesticides
Immune/autoimmune	Susceptibility to infections	Dioxins
	Autoimmune disease	Dioxins
Pulmono/cardiovascular	Heart disease/Hypertension	BPA
	Asthma	Air pollution

1.5.1 Reproductive effects

Most reproductive effects of EDCs are exerted through the disturbance of oestrogen and androgen-mediated processes. In laboratory rodents, exposure to BPA during prenatal, perinatal and adult stages of development, have been shown to cause many changes in the male reproductive system, including genitourinary abnormalities, decreased epididymal weight, decreased sperm production and increased prostate weight (Richter *et al.,* 2007; Knez, 2013). These changes were observed, even when exposure was limited to levels below NOAEL. BPA levels in blood have also been associated with a variety of conditions in women, including endometrial hyperplasia, endometriosis, recurring miscarriages and polycystic ovarian syndrome (PCOS) (Vandenberg *et al.,* 2007). Recently, a relationship between BPA levels and reproductive hormone levels in male patients, and the amount of oocytes retrieved

from women undergoing IVF was established (Mok-Lin *et al.,* 2009; Meeker *et al.,* 2010).

1.5.2 Neuro-endocrine effects

Thyroid function is a highly regulated negative feedback system of thyroid hormones circulating at hypothalamic and pituitary levels (Feldt-Rasmussen *et al.,* 1980). BPA has been shown to inhibit thyroid-receptor mediated transcriptional activity by binding to thyroid hormone receptors (Boas *et al.,* 2012). Exposure may also be implicated in the expression of thyroid related genes in the brain. A study has shown in a mouse model that the prenatal exposure to low doses of BPA affects the morphology and the expression of some genes related to brain development in the murine foetal neocortex (Nakamura *et al.,* 2006). Prenatal BPA exposure has also been implicated in increased externalised behaviours, such as hyperactivity and aggression (Vandenberg *et al.,* 2010).

1.5.3 Immunomodulatory effects

Many EDCs have been shown to have immunosuppressive effects. BPA and phthalates, for example, may stimulate immune responses at low concentrations. However, at higher doses, this response is attenuated resulting in a non-linear, inverted U-shape dose response (Kuo *et al.,* 2012).

BPA has been implicated in alterations to the biology of immune cells and exposure to BPA may contribute significantly to the initiation or cessation of inflammatory conditions. Furthermore, exposure to BPA may infer chronic inflammation, which in

turn, can cause cellular and tissue damage (Manabe, 2011). Selected immunomodulatory effects caused by BPA is summarised in Table 1.3.

1.5.4 BPA, obesity and diabetes

Exposure to various chemicals during prenatal development has been linked to increased weight and the occurrence of metabolic disorders, such as diabetes (Schug *et al.,* 2007). BPA has been shown to have similar effects. For example, human and mouse pre-adipocytes and adipocytes both express nuclear oestrogen receptors (ERα and ERβ). Exposure to BPA during the foetal and neonatal developmental stages has been shown to increase rate of postnatal growth and obesity in mice and rats (Heindel and vom Saal, 2009). In addition, BPA was shown to increase glucose transport in pre-adipocytes, and in combination with insulin caused an increase in the conversion of mouse fibroblasts into adipocytes, whilst also increasing lipoprotein lipase activity and triacylglycerol accumulation (Masuno *et al.,* 2002; Sakurai *et al.,* 2004).

1.6 EDCs in South African wastewaters

Prior to 1999, research on the possible contamination of EDCs in South African wastewaters was limited (Burger, 2008). Recent studies carried out in the U.S., Canada, Europe, Japan, Australia and South Africa, showed that over 50 potential EDCs may be present in the drinking water supply sources (AWWA, 2002). The first local histological evidence of intersex in feral sharptooth catfish from oestrogen-polluted water was reported by Barnhoorn *et al.* (2004).

Table 1.3: Cells of the immune system that are affected by exposure to BPA.

Immune cells affected	Description of impact on immune functions	Reference
CD4+ T-lymphocyte differentiation **(*T-helper 1 (Th1) cells – pro-inflammatory)** **(*T-helper 2 (Th2) cells – anti-inflammatory)**	Male mice that were fed low concentrations of BPA showed a biased production of Th1 cells. A Th2-biased outcome was observed when the mice were fed high concentrations of BPA (as high as 50 µM)	Youn *et al.,* 2002 Lee and Lim, 2010
Regulatory T-lymphocytes **CD4+CD25 cells (ensures that pro-inflammatory response returns to homeostasis)**	A reduction in the amount of regulatory T-lymphocytes was observed when mice were exposed to BPA either in prenatal stages or during adulthood. Prenatal exposure had a greater effect on the reduction of regulatory T-lymphocytes.	Yan *et al.,* 2008
B-lymphocytes	An increased production of immunoglobulin (Ig) A and IgG2a was observed in mice that underwent prenatal BPA exposure. In a murine model of the autoimmune disease, systemic lupus erythematosus, BPA increased the amount of B-lymphocytes producing autoantibodies.	Goto *et al.,* 2007 Yurino *et al.,* 2004
Macrophages	BPA (50 µM) inhibited the elevation of macrophages resulting from lipopolysaccharide activation. Macrophages isolated from mice that were orally treated with high concentration of BPA, showed suppressed production of tumour necrosis factor-alpha (TNF-α).	Kim and Jeong, 2003 Byun *et al.,* 2005

Mahomed *et al.* (2008) tested water from seven sites in the Pretoria West area (South Africa) which are surrounded by a significant number of small-sized industries considered to be potential sites of EDC pollution. The authors screened for oestrogenicity using the Recombinant Yeast Cell Bioassay (RCBA). Their study found that oestrogenic activity was detected in all the samples that were tested, which include compounds such as *p*-nonylphenol, bisphenol A and PCBs (Mahomed *et al.,* 2008). These findings reiterated the need for investigation of cost-effective treatment technologies for the removal of oestrogenic compounds from potential water sources.

1.7 Mechanisms for the removal of EDCs

The active removal of EDCs from water sources is an urgent priority, in order to ensure that the long-term effects of exposure to EDCs are further prevented. The following section presents selected physical, chemical and biological removal strategies currently being used. Particular emphasis is given to the use of laccase and the encapsulation of laccases that were employed in this study.

1.7.1 Activated Carbon

Using activated carbon (AC) for the removal of organic contaminants in wastewater is a well-known process. It is commonly employed in two forms, powder activated carbon (PAC) or as granular activated carbon (GAC). Studies on the removal of EDCs using AC typically focus on the removal efficiency of EDCs in different water systems, the type of carbon that is used and the physicochemical properties of the EDCs in question (Liu *et al.,* 2009). Kim *et al.* (2002) reported that the use of PAC for the removal of BPA might not be considered a good alternative to conventional methods

such as coagulation, flocculation and sedimentation of the EDC, due to its low removal efficiency (25-40% removal of BPA with a contact time of 15 minutes). GAC, on the other hand, was effective at removing BPA from wastewater when tested under the same conditions as PAC (Kim *et al.,* 2002). The effects of GAC types and service life were investigated for the removal of BPA, nonylphenol and amitrol (Choi *et al.,* 2005). GACs produced from coal, coconut and wood were tested. GAC was able to remove the EDCs with high water-octanol coefficient (K_{ow}) values (i.e. nonylphenol and BPA). All types of carbon tested effectively adsorbed nonylphenol and BPA, with better adsorption observed for nonylphenol, due to the lower K_{ow} value for BPA, when compared to nonylphenol. Compared to other types of GAC, the highest BPA adsorption was observed for coal-based carbon, which is attributed to its larger pore volume (Choi *et al.,* 2005). To its disadvantage, however, AC only adsorbs BPA and does not degrade it. Furthermore, the adsorption efficiency of AC varies greatly when comparing the treatment of simulated wastewater to real wastewater (Liu *et al.,* 2008).

1.7.2 Advanced Oxidation Processes

Chemical oxidation of EDCs by ozone, potassium permanganate and titanium oxide photocatalysis under ultraviolet (UV) radiation have been shown to successfully remove EDCs from wastewater (Liu *et al.,* 2009). To further enhance the removal efficiency of EDCs by photocatalysis, researchers often combine the use of direct photolysis with the inclusion of a chemical oxidant. Rosenfeldt and Linden (2004) demonstrated high removal efficiencies of BPA by combining photolysis by UV radiation with the chemical oxidant, hydrogen peroxide (H_2O_2). Combined UV/H_2O_2

photolysis resulted in up to 90% removal of BPA (Rosenfeldt and Linden, 2004). While these chemical oxidation methods are highly efficient, they are also prohibitively expensive, thus biological removal methods for the biodegradation of EDCs are a more cost-effective approach.

1.7.3 Biological removal of EDCs

A number of biological treatment methods have been described for the removal of micropollutants. Of these, activated sludge biological treatments are perhaps the cheapest available technology available for the removal and degradation of environmental contaminants (Esplugas *et al.,* 2007; Liu *et al.,* 2008). However, in the case of micropollutants, these compounds are not completely removed by activated sludge treatment. In addition, some of these micropollutants are adsorbed onto activated sludge and would require additional treatment for complete bioremediation (Johnson and Sumpter, 2001; Carballa *et al.*, 2004).

The degradation of BPA by microorganisms has been widely reported. Bacteria and fungi have been isolated from different sites and have been shown to exhibit BPA degrading activities (Chouhan *et al.,* 2013). A high degree of BPA degradation is attributed to the cellular processes of these organisms. For example, the degradation of BPA by *Sphingomonas* sp. strain AO1 was determined to be as a result of the action of cytochrome P450 (Sasaki *et al.,* 2005). On the other hand, BPA degradation can be attributed to the action of a specific group of enzymes.

Oxidative enzymes including laccases, tyrosinases and peroxidases catalyse the oxidation of a wide variety of hydroxyl- and amine-containing aromatic compounds (Kim and Nicell, 2006). The use of enzymes for the treatment of EDCs in wastewater is advantageous for many reasons, including high substrate specificity, they are active under a wide variety of pH and temperature conditions, and the simplicity of process control (Gassara *et al.,* 2013). Nevertheless, the use of free enzymes does have some limitations, such as high cost of production and purification of the enzymes, and lower stability of the catalysts in their free form (Aitken and Heck, 1998).

1.8 Laccases

Laccases are multicopper oxidases that catalyse the one-electron oxidation of four reducing substrate molecules, whilst concomitantly using molecular oxygen as an electron acceptor, converting it to water (Giardina *et al.,* 2010). Laccases usually consist of three cupredoxin domains (Figure 1.3A), although, two-domain laccases such as the bacterial laccase from *Streptomyces coelicolor* have also been identified (Figure 1.3B). Typically, laccase active sites contain four copper atoms: one type-1 copper (T1), one type-2 copper (T2) and two type-3 coppers (T3) with the T2 and T3 coppers forming a trinuclear cluster (Dwivedi *et al.,* 2011). Laccases have a broad substrate range, as they are able to oxidise a variety of substrates, including substituted phenols, diamines, aromatic amines and thiols (Dwivedi *et al.,* 2011). The majority of laccases of fungal origin are optimally active at a pH range of 3.5 – 5.0, while many bacterial laccases show activity at a higher pH (Reiss *et al.,* 2011). Laccases are attractive alternatives for application in the removal of organic pollutants in

wastewater treatment systems due to their ability to operate under a wide variety of conditions with a broad substrate range.

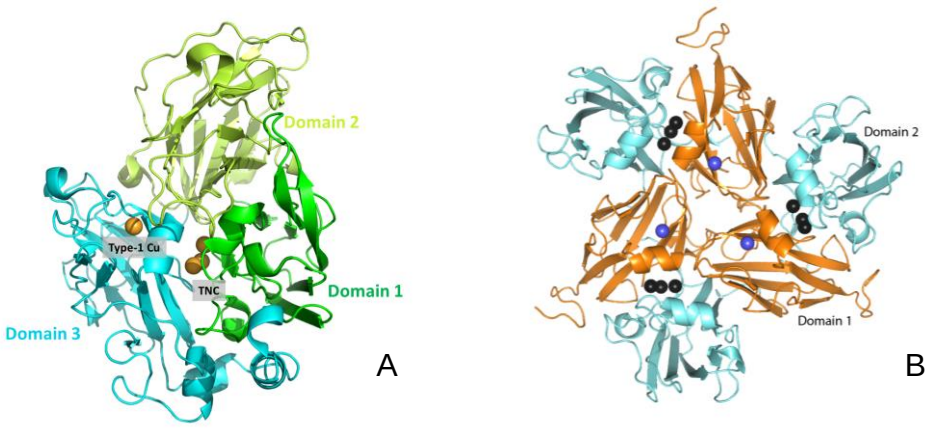

Figure 1.3: 3-D protein models of a typical monomeric three-domain laccase (A) from *Trametes versicolor* (PDB ID: 1GYC) and a homotrimeric two-domain laccase (B) from *Streptomyces coelicolor* (PDB ID: 3CG8)

1.8.1 Laccases and mediators

Laccases are unable to oxidise substrates of high complexity (such as lignin) or that have a higher redox potential than that of the enzyme. These limitations have led to an increase in a range of compounds discovered that can be used as the Laccase Mediator System (LMS) (Madhavi and Lele, 2009). Mediators are compounds that act as intermediate substrates for the laccase, generating reactive radicals allowing for the indirect oxidation of substrates with high redox potential (Figure 1.4) (Rodriguez-Couto and Herrera, 2006). Many natural and synthetic mediators have been investigated; common mediators include 1-hydroxybenzotriazole (1-HBT), violuric acid (VLA), ferulic acid, vanillin and 3-hydroxyanthranilic acid (3-HAA) (Dwivedi *et al.,* 2011; Figure 1.5). Due to the instability, toxicity and higher cost of synthetic

mediators, the use of natural mediators, such as vanillin and ferulic acid, is increasingly being investigated (Alcalde, 2007).

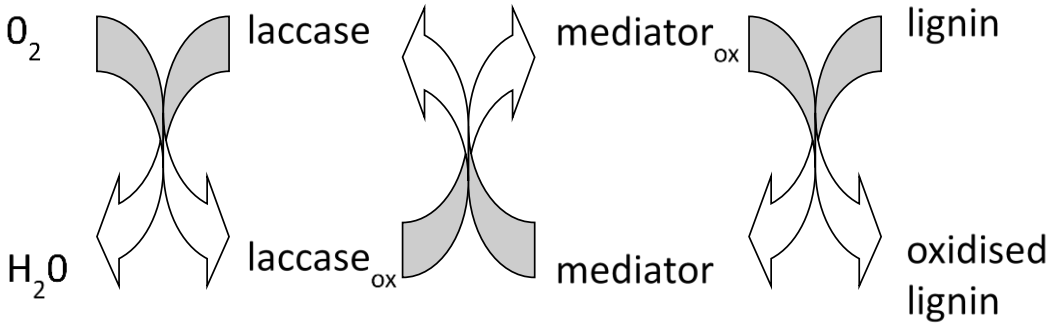

Figure 1.4: Schematic of mediator-assisted oxidation of lignin by laccase (modified from Bourbonnais *et al.,* 1998)

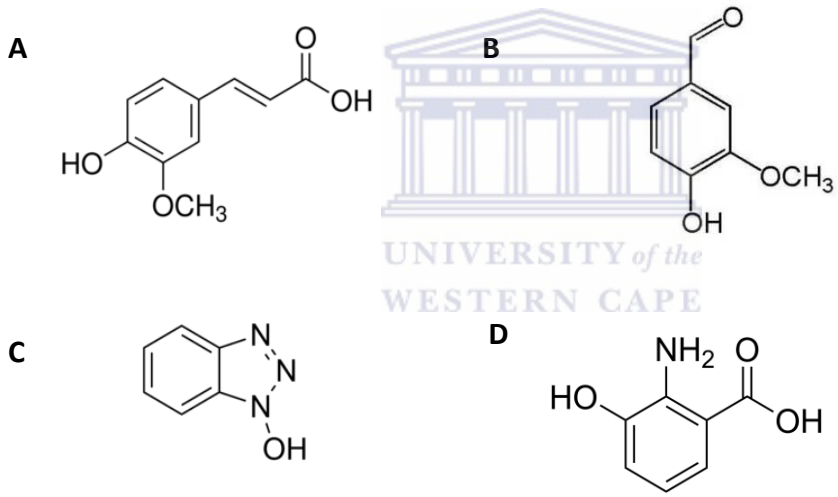

Figure 1.5: The chemical structures of commonly used laccase mediators: A – ferulic acid; B – vanillin; C – 1-hydroxybenzotriazole; D – 3-hydroxyanthranilic acid

1.8.2 The use of free laccase for the removal of BPA

There is extensive literature on the use of laccases for the treatment of EDCs in wastewater. Many studies have used enzyme preparations from a variety of fungal strains for the removal of BPA. The laccase from *Coriolopsis cinereus* completely removed 5 mg/L BPA from solution within four hours. More importantly, the

oestrogenic activity of the resultant metabolites was reduced by 80% (Cabana *et al*., 2007a). Similar results were noted for the laccase from *Trametes versicolor,* with a high removal efficiency of 99.9% and only 0.9% of toxicity compared to the starting material (Kim and Nicell, 2006). However, high removal efficiencies are not only observed for laccases of fungal origin. An extracellular bacterial laccase produced from *Pseudomonas* sp. strain LBC1 was purified and used for the removal of BPA. LCB1 laccase was able to completely biodegrade BPA (initial concentration of 36 mg/L) within five hours without the requirement of a redox mediator (Telke *et al.,* 2009).

1.8.3 Immobilisation of laccase for the removal of BPA

Whilst laccases in their free form have shown immense promise for the degradation of BPA, it is not feasible for industrial applications such as the treatment of wastewater, mainly because: 1) enzymes are unstable due to varying environmental conditions, and 2) free enzymes cannot be recovered from large volumes. It is therefore essential to employ an immobilisation strategy that will allow the enzymes to remain stable during the processing of the wastewater, while ensuring that the enzyme can be recovered for repeated use (Cabana *et al.,* 2009).

The most common method of immobilisation of laccase for the treatment of EDCs is the covalent immobilisation of laccase onto a solid support. This immobilisation strategy involves the formation of covalent bonds between amino acids that are not involved in the activity of the enzyme and surface groups of the solid support. Examples of supports include AC, silica beads, carbon nanotubes, and gold and silver nanoparticles (Cabana *et al.,* 2009; Fernandez-Fernandez *et al.,* 2012). Covalently

immobilised laccase has been successfully used for the treatment of EDCs. Laccase from the white rot fungus, *Coriolopsis polyzona,* was covalently immobilised onto diatomaceous earth support (Celite®) R-633. The complete removal of BPA (5 mg/L) was achieved after five consecutive cycles of 200 minutes contact time per cycle. BPA removal of up to 70% was achieved using the same retention time of 200 minutes and an increased concentration of BPA (100 mg/L) (Cabana *et al.,* 2009). Catapane *et al.* (2013) immobilised laccase from *T. versicolor* onto polyacrylonitrile (PAN) beads for the removal of two alkylphenols (APs), octylphenol (OP) and nonylphenol (NP). The immobilised laccase was able to remove both EDCs, albeit at retention times that varied by 15%.

Furthermore, the use of cross-linked enzyme aggregates (CLEAs) have been used as an immobilisation strategy for the treatment of EDCs. This method of self-immobilisation removes the requirement for a solid support, and CLEAs may exhibit robust operational stability and enzyme activities (Fernandez-Fernandez *et al.,* 2012). Cabana *et al.* (2007b) prepared CLEAs from laccase isolated from *C. polyzona* and used it for the elimination of EDCs. The CLEAs were packed in a fluidised bed reactor and completely removed BPA from the treated solution (Cabana *et al*., 2007b).

The formation of cross-linked enzyme crystals (CLECs) are a variation on the approach to making CLEAs. CLECs are produced through a two-step process. The first step involves the batch crystallisation of the enzyme (which typically requires an enzyme of high purity). This is followed by the cross-linking of the crystals using a bi-functional agent, such as glutaraldehyde, in order to stabilise the proteins, retain enzyme activity

and maintain the crystal lattice (Margolin, 1996; Govardhan, 1999). The formation of enzyme crystals allows the enzyme to remain active in otherwise harsh conditions, such as prolonged exposure to high temperatures, aqueous-organic solvent mixtures, extended exposure to high temperatures and exogenous protein degradation (Persichetti *et al.,* 1995; Govardhan, 1999).

The use of laccase CLECs for the degradation of EDCs has not previously been reported. Roy *et al.* (2005), however, reported the use of CLECs from *T. versicolor* laccase as a biosensor. The CLECs were highly active towards a number of phenolic compounds and were able to retain good activity for over 3 months (Roy *et al.,* 2005), demonstrating that they may be advantageous for the degradation of phenolic EDCs, such as BPA.

1.8.4 The removal of oestrogenic activities of EDCs using laccases

Ultimately, the goal of any process that is aimed at the treatment of toxic environmental pollutants should be to either completely remove the contaminant or degrade it to less toxic alternatives. Quite a few studies that focused on the degradation of BPA using either free or immobilised laccase, also assessed whether the metabolites produced through enzymatic degradation yielded compounds with altered oestrogenicity.

Oestrogenic activity can be determined by whole organism assays and cellular bioassays. Whole organism assays make use of the process of endocrine disruption in amphibians, fish, birds and insects. The response of these organisms is determined by

looking at deformities, reproductive deficiencies, eggs and offspring production (Chang *et al.,* 2009). Cellular bioassays, on the other hand, determine the oestrogenic activity of a compound through a protein expression system, which represents the oestrogen response stimulated by the binding of oestrogen to the oestrogen receptor. The response is monitored by quantification of response proteins included in the bioassay, such as luciferase and β-galactosidase, which can be monitored with a luminometer or a spectrophotometer, respectively (Campbell *et al.,* 2006; Houck and Kavlock, 2008).

Cabana *et al.* (2007a) investigated the degradation of BPA and NP using a fungal laccase from *C. polyzona.* In addition to confirming high removal rates for both BPA and NP, it was determined that there was a loss in oestrogenic activity when the spent solution was tested through the use of the Yeast Oestrogen Screens. The loss of oestrogenicity is thought to be linked to the formation of high molecular weight compounds that are unable to bind to the human oestrogen receptor (Cabana *et al*., 2007a). Similar results have been reported by Saito *et al.* (2004) where they showed complete removal of oestrogenic activity for both BPA and NP within 24 hours when samples were treated with an extracellular fungal laccase from *Chaetomiaceae* sp. strain I-4. The work performed by Catapane *et al.* (2013) also demonstrated that laccase immobilised on PAN beads completely removed the oestrogenic activity of solutions containing OP and NP.

It is interesting to note that many of these studies have been performed using laccases of fungal origin. While many bacteria capable of degrading BPA have been isolated,

most of them are not efficient at degrading BPA (Sasaki *et al.,* 2005). Oshiman *et al.* (2007) demonstrated the use of *Sphingomonas bisphenolicum* strain AO1 for the treatment of BPA which led to an initial increase of oestrogenic activity (3.6 times of BPA) before complete removal of oestrogenic activity.

1.9 Objectives of this study

In this study, the removal of BPA from aqueous solutions was assessed, comparing the removal efficiencies between two fungal laccases (*T. versicolor* and *Trametes pubescens*) and two bacterial laccases (isolated from *S. coelicolor* and *Escherichia coli*). A mutant laccase variant of the laccase from *S. coelicolor* (SLAC-VN), which was designed in a previous study, was also used. The treatment of BPA was investigated using two approaches:

- The first approach entailed the use of the free laccase enzymes from each organism for the removal of BPA from aqueous solution under each enzyme's optimal conditions.
- The second approach entailed using two immobilisation strategies: the formation of CLECs, CLEAs, and the encapsulation of laccase in sodium alginate beads.

The removal efficiency for the two approaches was assessed and the metabolites generated, analysed by high performance liquid chromatography and mass spectrometry. In addition, the effect of two natural and two synthetic laccase mediators on the degradation efficiency of the laccases was also assessed.

The following chapter will cover the materials and methods used during this study. This will be followed by the results and discussion (chapter three), and a final summative discussion in chapter four.

CHAPTER TWO

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich, Merck Millipore and Fermentas, unless otherwise stated. Prof. Gerard Canters (Leiden Institute of Chemistry, University of Leiden, The Netherlands) kindly provided the plasmid containing the gene encoding for SLAC. The SLAC mutant, SLAC-VN, was generated by Ms. Leela Kleinsmidt (Prins *et al.,* 2015). Five laccases were used in this study. Three of the laccases were produced in-house – the laccases from *Trametes pubescens, Streptomyces coelicolor* and the SLAC mutant, SLAC-VN, and the copper-transport protein, CueO from *Escherichia coli*. Laccase from *Trametes versicolor* (TvL) was purchased from Sigma-Aldrich. TvL was prepared by resuspending 0.5 mg of enzyme per mL of 100 mM sodium acetate buffer (pH 5.0)

2.1 Enzyme Production and Purification

2.1.1 Production and purification of SLAC, SLAC-VN and CueO

Overnight cultures were prepared by inoculating 50 µL of *E. coli* BL21 containing pET-SLAC, pET-SLAC-VN or pET-CueO in 5 mL 2xYT (Appendix A) supplemented with 100 µg/mL filter-sterilised ampicillin. The cultures were incubated overnight at 37°C, shaking at 160 rpm on an orbital shaker. Two mL of the overnight culture was transferred to 200 mL 2xYT media (containing 100 µg/mL ampicillin) and allowed to proliferate until an OD_{600nm} measurement of 0.8 on a spectrophotometer (Perkin-Elmer) was reached. Overexpression of the laccases was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM once

the OD_{600nm} reached 0.8. The culture was then incubated overnight at 22°C; shaking at 160 rpm. An uninduced control was also prepared in the same manner with the omission of IPTG for each enzyme variant.

Cells were harvested by centrifugation at 5000 *xg* for 10 minutes using a Multifuge 3SR+ centrifuge (Thermo Scientific). Cell pellets were resuspended in 2.5 mL of ice-cold 10 mM potassium phosphate buffer (pH7.3). The resuspended cells were sonicated (on ice) for a total of 5 minutes (15s bursts followed by 15s rests) using a United Scientific sonicator. The soluble fractions were collected by centrifugation at 13 500 *xg* for 40 minutes. The soluble fraction of both induced and uninduced samples was incubated overnight at 4°C with 1 mM $CuSO_4$, 0.3 U/mL DNAse I (Fermentas) and 0.3 U/mL RNAse I (Fermentas). The samples were dialysed against 10 mM potassium phosphate buffer (pH7.3) using SnakeSkin Dialysis Tubing (ThermoScientific; MWCO 10 000 Da) for four dialysis sessions (each session for 1 hour; 1 mM EDTA was added during the second session to chelate excess copper ions).

A 75 mL size-exclusion column was prepared by heating 8.5 g of Sephadex G-75 beads at 90°C for 2 hours in order for the beads to swell. The bead slurry was cooled and poured into a pre-rinsed (with 10 mM potassium phosphate) glass chromatography column. After allowing the beads to set, the column was equilibrated with three column volumes of 10 mM potassium phosphate (pH 7.3). The induced laccase samples were applied to the size-exclusion column. Fractions of 1 mL were collected by gravity elution when the blue band (representing the presence of enzyme) of the samples neared the end of the column. Fractions were analysed for protein

concentration (Bradford's Protein Assay) and laccase activity [2,6-dimethoxyphenol assay (2,6-DMP)]. Fractions with the highest specific activity were combined and the final specific activity was determined. Specific activity is defined here as the amount of enzyme required to convert 1 micromole of substrate per minute per milligram of protein (µmol/min/mg) at the conditions of the experiment.

The purification of the bacterial laccases, SLAC and SLAC-VN was also performed using fast performance liquid chromatography (FPLC) using an AKTAprime™ system (Amersham-Biosciences). Five mL of enzyme sample was applied to a 120 mL HiLoad 16/60 Superdex 75 chromatography column (Sigma-Aldrich). Separation of the samples was performed using a flow-rate of 0.100 mL/min and 10 mM potassium phosphate (pH 7.3) as the mobile phase. Fractions of 2 mL were collected, and the fractions were analysed for laccase activity and protein concentration. Fractions with the highest specific activity were combined and the final specific activity was determined.

2.1.2 Production and purification of laccase from *Trametes pubescens*

A fresh culture of *T. pubescens* #696.94 (CBS, Netherlands) was grown on 3% (w/v) malt extract agar (28°C; 5 days). One half of an agar plate containing *T. pubescens* was homogenised in a sterile Waring blender. The homogenised culture was aseptically inoculated into 200 mL *Trametes* Defined Medium (Appendix A). The culture was incubated for 3 days at 28°C, shaking at 175 rpm on an orbital shaker. On the third day, laccase production was induced by adding 1.82 mL of an induction mixture

(mg/30 mL: 234.0 phenol; 84.0 *p*-cresol). Laccase activity was monitored for 2 days before harvesting.

The supernatant of the culture was harvested by centrifugation at 12 086 *xg* for 10 minutes. Ammonium sulphate to 50% saturation was added to the supernatant, and the sample was stirred gently for 30 minutes at 22°C. The protein precipitate was pelleted by centrifugation at 10 000 *xg* for 10 minutes at 4°C. The pellet was resuspended in 0.1x the initial volume in 100 mM sodium acetate buffer (pH 5.0). Both the supernatant and pellet were tested for laccase activity and protein concentration. Ammonium sulphate was added to the supernatant to 80% saturation and the protein was precipitate as described above. The 80% pellet was resuspended in 0.1x the initial volume and dialysed overnight against 100 mM sodium acetate buffer (pH5.0) at 4°C.

2.1.3 Determination of laccase activity

Laccase activity was routinely measured using 1 mM 2,6-dimethoxyphenol (2,6-DMP) at each enzyme's optimal pH (SLAC – pH 9.5; SLAC-VN – pH 7.5; TvL – pH 4.5; TpL – pH 5.0). All assays were performed in duplicate in microtitre format using a Thermoscientific Multiskan 1000 microtitre plate reader. 150 µL of substrate-buffer mix was added to 50 µL of enzyme sample. Substrate oxidation was monitored by the change in absorbance and was measured for 2 minutes.

For the copper-dependant CueO, laccase activity was measured as described above, except using 1 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as substrate at pH 5.0 in the presence of 1 mM $CuSO_4$. ABTS was used as a known laccase substrate for CueO, as reported by Zeng *et al.* (2011).

Enzyme activity was determined through the use of the following formula:

$$\frac{\mathbf{U}}{\mathbf{ml}} = \frac{\frac{\mathbf{\Delta Abs}}{\mathbf{min}}}{\boldsymbol{\varepsilon}} \times \frac{\mathbf{total\ reaction\ volume}}{\mathbf{enzyme\ volume}} \times \mathbf{dilution\ factor}$$

where:

ΔA/min is the change in absorbance per minute

ε is the extinction coefficient of the substrate in $mM^{-1}\,cm^{-1}$

dilution factor dilution of sample (if sample was diluted)

2.1.4 Protein quantification

A 1 mg/mL stock solution of bovine serum albumin (BSA) was prepared by dissolving 10 mg crystalline BSA in 10 mL 10 mM potassium phosphate buffer (pH 7.3) or 100 mM sodium acetate buffer (pH 5.0). Working standards for the standard curve were prepared from 100 µg/mL BSA, as described in Table 2.1.

Table 2.1: Preparation of standards used in Bradford's Protein Assay

Standard	Vol. 100 µg/mL BSA (µL)	Vol. buffer (µL)	BSA conc. (µg/mL)
Blank	0	100	0
1	10	90	10
2	20	80	20
3	40	60	40
4	60	40	60
5	80	20	80
6	100	0	100

In a 96-well microtitre plate, 20 µL of each standard/sample was aliquotted per well (in duplicate) and 180 µL of Bradford's Reagent was added. The reaction was left to stand for 2 minutes and the absorbance measured at 595 nm using a Thermoscientific

Multiskan 1000 microtitre plate reader. A standard curve was constructed by plotting absorbance of standards versus their respective concentrations. The concentration of each sample was estimated by using the regression line equation from the standard curve (Appendix A).

In addition, the protein concentration of the enzyme sample was determined in order to calculate the specific activity, which was calculated using the following equation:

$$\frac{\mathbf{U}}{\mathbf{mg}} = \frac{\mathbf{U}}{\mathbf{mL}} \div \frac{\mathbf{mg}}{\mathbf{mL}}$$

where:

U/mg is the specific activity

U/mL is the enzyme activity

mg/mL is the protein concentration

2.1.5 SDS-PAGE analyses

A 5% stacking gel and 12.5% resolving gel was used for the separation of the proteins of interest. The gels were poured as indicated in the manufacturer's instructions for the BIO-RAD PROTEAN Mini-Gel system. The reagents required were prepared as follow:

i. Resolving gel buffer (1.125 M Tris-HCl, pH 8.8 and 0.3% SDS)

For 200 mL: 27.25g Tris was dissolved in 150 mL distilled water, the pH adjusted to 8.8 with HCl, 0.6g SDS was added and made up to 200 mL with water. The solution was autoclaved for 20 minutes, 15 psi.

ii. Stacking gel buffer (0.375 M Tris-HCl, pH 6.8 and 0.3% SDS)

For 200 mL: 9.08g Tris was dissolved in 150 mL distilled water, the pH adjusted to 6.8 with HCl, 0.6g SDS was added and made up to 200 mL with water. The buffer was autoclaved for 20 minutes, 15 psi.

iii. For the 12.5% resolving gel, the following solutions were mixed prior to pouring the gel: 4.17 mL acrylamide/bis-acrylamide (29.2g:0.8g), 2.5 mL resolving gel buffer, 3.33 mL distilled water, 50 µL 10% ammonium persulphate, and 15 µL TEMED. The resolving gel was covered with a layer of isopropanol to allow the gel to set. Prior to pouring the stacking gel, the isopropanol layer was removed.

iv. For the 5% stacking gel, the following solutions were mixed and poured onto the resolving gel: 1.34 mL acrylamide/bis-acrylamide mix (29.2g:0.8g), 2.0 mL stacking gel buffer, 4.6 mL distilled water, 60 µL 10% ammonium persulphate, and 15 µL TEMED. The comb was inserted into the stacking gel and the gel was allowed to set.

Twenty µL of each laccase sample was mixed with 5 µL of 5x sample buffer containing dithiothriotol (DTT) [3.1 mL 1 M Tris-HCl (pH 6.8), 5 mL glycerol, 0.5 mL bromophenol blue, 5 mM DTT, and 1.4 mL water]. The 25 µL samples were boiled for 10 minutes and loaded onto the gel. The gel was run at 180V for 45 minutes in electrophoresis buffer [mix 3.0 g Tris (25 mM), 14.4g glycine (192 mM) and 1g SDS in 1 litre water; the final pH should be 8.8] at room temperature (22°C). The gel was stained overnight

with Fermentas PageBlue Coomassie on a rocking shaker. Destaining was performed using distilled water for 20 minutes.

2.2 Enzyme immobilisation

2.2.1 Encapsulation of laccase using sodium alginate

The two fungal laccases (TvL and TpL) and two bacterial laccases (SLAC and SLAC-VN) were encapsulated into sodium alginate beads following the methods described by Bashan (1986) and Fraser and Bickerstaff (1997). For each laccase, 10 units of enzyme were mixed with sodium alginate to final alginate concentrations of 1%, 2%, 3% and 4% (w/v), in a total reaction volume of 4 mL. The enzyme-alginate mixtures were gently agitated on a rotating shaker at 40 rpm for 1 hour at 4°C. The enzyme-alginate mixture was dropped from a height of 5 cm (using a luer-slip syringe and needle) into ice-cold calcium chloride (2%) to form the beads. The beads were allowed to cure at 4°C for 16 hours prior to use. Residual unbound laccase was washed off with sterile distilled water. Wash fractions were tested for laccase activity in order to determine the amount of laccase that was encapsulated.

The loading efficiency was calculated based on the total amount of unbound enzyme using the following equation:

$$\textbf{Loading efficiency} = \frac{\textbf{(A-B)}}{\textbf{A}} \times \textbf{100}$$

where:

A the total amount of enzyme added

B the amount of enzyme unbound

2.2.2 Production of cross-linked enzyme crystals (CLECs)

CLECs were produced by following the method adapted from Roy and Abraham (2006). Enzyme crystals were prepared by the addition of ammonium sulphate to 1 mL of laccase (in optimal storage buffer) to a final concentration of 75%. The ammonium sulphate was dissolved to saturation by stirring for 3 hours at 4°C. The saturated enzyme solutions were kept undisturbed at 4°C for 20 hours. The crystals were separated by centrifugation at 483 *xg* for 8 minutes (at 4°C). The pelleted crystals were washed with 100% isopropanol in order to remove excess ammonium sulphate.

Cross-linking was performed by adding to 2 mL of glutaraldehyde (1.5% in isopropanol) to the pelleted crystals. The crystals and glutaraldehyde were mixed using a carousel at 15 rpm for 20 minutes at room temperature (22°C). The crystals were washed twice with optimal storage buffer (100 mM sodium acetate, pH 5.0 for TvL and TpL; 10 mM potassium phosphate, pH 7.3 for SLAC and SLAC-VN). The crystals were stored in optimal storage buffer at 4°C until use. The isopropanol and washes were kept for analyses to determine immobilisation efficiency.

2.2.3 Production of cross-linked enzyme aggregates (CLEAs)

The method for the preparation of cross-linked enzyme aggregates (CLEAs) was adapted from Xu *et al.* (2011). The enzyme solutions were standardised at a concentration of 2 U/mL. For each laccase, 1 mL of enzyme solution was mixed with 50 mg of BSA prior to the addition of ammonium sulphate to a final concentration of 80% and allowed to mix on a carousel (15 rpm) at room temperature (22°C). One set was prepared without the addition of BSA. Glutaraldehyde was added to a final

concentration of 1, 2 or 3%. The solutions were allowed to mix for 3 hours on a carousel (15 rpm) at room temperature (22°C). The solutions were centrifuged at 13 000 *xg* for 15 minutes at 4°C. The supernatant was removed and kept for enzyme analysis. The pellets were washed with sterile distilled water and dispersed in buffer (100 mM sodium acetate, pH 5.0 for the fungal laccases; 10 mM potassium phosphate for SLAC and SLAC-VN) and stored at 4°C until use. Laccase activity was measured by dispersing 50 µL of CLEAs and allowing it to react with 1 mM ABTS in ammonium acetate buffer (pH 4.0 for the bacterial laccases and pH 5.0 for the fungal laccases).

2.3 BPA removal experiments

All experiments were set up in duplicate, unless otherwise stated.

2.3.1 Removal of BPA using free laccase

2.3.1.1 pH optimisation

The method used for the removal of BPA was adapted from Telke *et al.* (2009) and was set-up according to Table 2.2. A typical 750 µL reaction consisted of 1 U/mL of enzyme (TvL, TpL, SLAC and SLAC-VN), 20 mM buffer (ammonium acetate at pH 3.0 and 5.0; ammonium bicarbonate at pH 7.0 and 9.0) and 10 mg/L of BPA. The reactions were incubated on a carousel at 45 rpm for 16 hours at 22°C. The reactions were also set-up at one pH value above and one pH value below the initially determined pH optimum (in 0.5 pH value increments) in order to narrow down the optimal pH for each enzyme.

Table 2.2: Sample preparation for EDC removal by free enzyme solutions

	Sterile water (µL)	40 mg/L BPA (µL)	50 mM buffer (µL)	5 U/mL enzyme (µL)
Substrate control	262.5	187.5	300	-
Enzyme control	300	-	300	150
Replicate 1	112.5	187.5	300	150
Replicate 2	112.5	187.5	300	150

2.3.1.2 Temperature optimisation

In order to determine the optimum temperature for the removal of BPA, reactions were prepared in the same manner as described in Table 2.2, using the optimal pH determined for each enzyme. The samples were incubated at 20°C, 30°C and 40°C for 16 hours.

2.3.1.3 Optimisation of reaction time

Reactions were set up at optimal pH and temperature for each laccase as described in Table 2.2. The reactions were incubated for 2, 8 and 16 hours prior to HPLC analyses.

2.3.1.4 BPA removal in the presence of laccase redox mediators

Reactions were set up at optimal pH and temperature for 16 hours for each laccase in the presence of four known laccase mediators: two natural mediators, ferulic acid and vanillin, and two synthetic mediators, 1-HBT and TEMPO. Laccase mediators were tested at concentrations of 0.1 mM, 0.2 mM and 0.5 mM.

2.3.2 Removal of BPA using laccase encapsulated in sodium alginate beads

2.3.2.1 pH optimisation

The optimal pH for BPA removal by the immobilised laccases was determined by setting up experiments at pH 3.0, 5.0, 7.0 and 9.0. A typical reaction mixture contained: 20 mM buffer (ammonium acetate buffer at pH 3.0 and 5.0; ammonium bicarbonate buffer at pH 7.0 and 9.0); an amount of beads that equalled a theoretical value of 0.5 U of enzyme and 10 mg/L BPA. The samples were incubated for 16 hours at 22°C on a carousel at 45 rpm. pH optimisation was further narrowed down by performing the reactions at one pH value above and one pH value below the initially determined pH optimum (in 0.5 pH value increments).

2.3.2.2 Temperature optimisation

The optimal temperature for BPA removal using encapsulated laccase was performed at optimal pH, as described in section 2.3.2.1. The reactions were incubated at 20°C, 30°C and 40°C for 16 hours, prior to HPLC analyses.

2.3.2.3 Optimisation of reaction times

Reactions were set up at optimal pH and temperature for each laccase as described above. The reactions were incubated for 2, 8 and 16 hours prior to HPLC analyses.

2.3.2.4 BPA removal in the presence of redox mediators

Reactions were set up at optimal pH and temperature for 16 hours for each encapsulated laccase in the presence of the four known laccase mediators. Laccase mediators were tested at concentrations of 0.1 mM, 0.2 mM and 0.5 mM.

2.3.2.5 Reusability of encapsulated laccase

In order to determine whether the encapsulated enzyme retained sufficient activity after multiple uses, for each laccase, the reactions were set up under optimal pH and temperature conditions. The reactions were incubated for multiple cycles, where the duration of each cycle was 16 hours. After each cycle, the beads were removed, washed with sterile H_2O and added to a fresh reaction mixture.

2.3.3 Removal of BPA using cross-linked enzyme aggregates

2.3.3.1 pH optimisation

For each laccase, the optimal pH for the removal of BPA using CLEAs was determined by setting up the reactions at pH 3.0, pH 5.0, pH 7.0 and pH 9.0. A typical reaction consisted of 10 mg of CLEAs, 20 mM buffer (ammonium acetate pH 3.0 and pH 5.0; ammonium bicarbonate at pH 7.0 and pH 9.0) and 10 mg/L BPA. The reactions were incubated for 16 hours prior to HPLC analyses. The optimal pH was further optimised by setting up experiments at one pH value above and one pH value below the initially determined pH value.

2.3.3.2 Temperature optimisation

The optimal temperature for BPA removal using the CLEAs was performed at the optimal pH for each of the laccase CLEAs. The reactions were set up as described in section 2.3.3.1. The reactions were incubated at 20°C, 30°C and 40°C for 16 hours prior to HPLC analyses.

Furthermore, the effect of temperature on the integrity of the CLEAs was determined. After the 16 hours incubation as described above, the supernatants were analysed by SDS-PAGE in order to determine if any enzyme subunits have been released from the CLEAs.

2.4 High Performance Liquid Chromatography (HPLC) analysis

Prior to HPLC analysis, the reactions that were incubated for 16 hours were treated with an equal volume of ice-cold methanol (100%) and incubated on ice for 30 minutes to precipitate the enzyme. This was followed by centrifugation at 9 500 *xg* for 10 minutes and transferred to 1.5 mL HPLC vials for analysis. Additionally, for the encapsulated laccase, when product analysis by LC-MS was performed, the used alginate beads were dissolved in 100 mM potassium phosphate to release any potential products that were trapped in the beads.

Standard calibration curves for BPA were constructed by HPLC analysis of samples with a concentration range of 0.1-10 mg/L (Appendix B). Standards were prepared in 50% methanol. HPLC analysis was performed for the initial detection of BPA removal using a La-Chrom® D-7000 HPLC with a La-Chrom® L-7400 Ultraviolet Detector using the method adapted from Telke *et al.* (2009). Separation of the samples was carried out using isocratic elution on a Waters® Spherisorb C-18 reversed phase column (4.6 x 250mm). The mobile phase used was water and acetonitrile in a ratio of 40% water: 60% acetonitrile. A flow-rate of 0.5 mL/min and injection volume of 10 µL was used and the UV detector set at 277 nm. Sample acquisition time was set to 10 minutes.

BPA removal was calculated using the following equation:

$$\% \text{ BPA removal} = 100 - \left(\frac{\text{initial BPA concentration}}{\text{final BPA concentration}} \times 100\right)$$

2.5 Liquid Chromatography – Mass Spectrometry (LC-MS) analysis

Putative BPA metabolites were analysed for by LC-MS. Samples were prepared in the same manner as described in section 2.4. LC-MS analysis was carried out on a Dionex® Ultimate 3000 HPLC (Dionex Softron, Germany) that was equipped with 3000RS pump, WPS 3000RS autosampler and a DAD-3000RS diode array detector. The sample separation was carried out on a Waters® Sunfire C-18 reversed phase column (4.6 x 150 mm) using a linear gradient of 0.1% formic acid (solvent A) and 99% acetonitrile (solvent B). The gradient was set up as follows: 98% A – 2% A (20 min); 2% A – 98% A (20-21 min); 98% A (21-25 min). The flow-rate was 0.5 mL/min, the injection volume was 10 µL and the oven temperature was set to 30°C. The metabolites were monitored and detected at 277 nm. The HPLC was coupled to a Bruker ESI Q-TOF mass spectrometer (Bruker Daltonik GmbH, Germany). The products that were separated as described above were analysed in negative mode using the full scan and auto MS/MS (collision energy 25eV) scan modes with dual spray for reference mass solution. The electrospray voltage, dry gas flow and nebuliser gas pressure was set to +3500V, 8 L/min and 17.5 psi, respectively. Putative products were identified by analysing the mass spectra generated for each sample using Compass DataAnalysis software v4.0 SP4 (Bruker Daltonik, GmbH, Germany). Known metabolites of BPA were selected from literature and used as a reference point.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Enzyme Production and Purification

In this study, four laccases, two of fungal origin (*Trametes pubescens* – TpL and *Trametes versicolor* – TvL) and two bacterial laccases (*Streptomyces coelicolor* – SLAC and a mutant of the *S. coelicolor* laccase – SLAC-VN; and CueO from *Escherichia coli*) were selected to determine their ability to degrade BPA. TpL, SLAC, SLAC-VN and CueO were produced in-house. Laccase from *T. versicolor* was purchased from Sigma-Aldrich.

Prior to the inclusion of SLAC-VN in this study, the copper transport protein from *E. coli* was selected for investigation. However, due to the low laccase activity compared to the high amount of protein produced, and the inconsistency with the amount of laccase produced (0.97 U/mg ± 0.88) (Table 3.1), CueO was replaced with a SLAC mutant that was shown to have high specific activity compared to the unmutated SLAC (Prins *et al.,* 2015). Since very low activities were detected for CueO, it was decided to perform the BPA removal studies only with TpL, TvL, SLAC and SLAC-VN.

The laccases were purified by size-exclusion chromatography and ammonium sulphate precipitation for the SLAC variants and *T. pubescens* laccase, respectively and analysed by SDS-PAGE (Figure 3.1). Fungal laccases are typically homodimers of two monomers 50-70 kilodaltons (kDa) in size (Baldrian, 2006). This was confirmed by the presence of a band of approximately 100 kDa for both *T. versicolor* and *T. pubescens*

laccase (Figure 3.1, Lanes 2 and 3). Other bands are observed for the commercial laccase, TvL. The presence of other enzymes in the commercial preparation of TvL was reported in a previous study (Kirchner, unpublished data). The bacterial laccases, SLAC and SLAC-VN, were observed in their typically trimeric form at approximately 90 kDa. These enzymes are highly resistant to the denaturing conditions when performing SDS-PAGE analysis (Prins *et al.,* 2015).

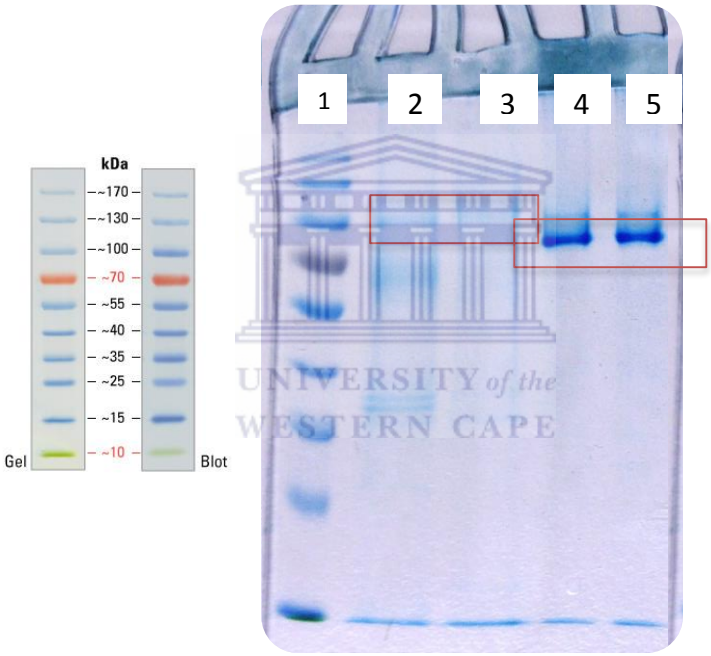

Figure 3.1: SDS-PAGE of laccases used in this study. Lane 1 – Molecular Weight Marker; Lane 2 – Laccase from *T. versicolor*; Lane 3 – Laccase from *T. pubescens*; Lane 4 – Laccase from *S. coelicolor*; Lane 5 – The SLAC mutant, SLAC-VN

The fungal laccase, TpL, exhibited a significantly higher specific activity when compared to the bacterial laccases (600 U/mg compared to less than 1.8 U/mg) (Table 3.1). The large difference in specific activity can be attributed to the higher redox potential at the T1 copper, which is typical for fungal laccases (Xu *et al.,* 1998; Baldrian, 2006). As a result of the large difference in specific activity between the laccases,

enzyme concentrations used in this study was standardised using enzyme activity (U/mL), rather than specific activity.

Table 3.1: The specific activities (U/mg) of the laccases produced in this study. The results presented are averaged from two expressions (mean ± standard deviation)..

	Specific activity (U/mg)
***T. pubescens* laccase**	600.07 ± 200.52
***S. coelicolor* laccase (SLAC)**	1.72 ± 0.27
***S. coelicolor* laccase mutant (SLAC-VN)**	0.71 ± 0.15
***Escherichia coli* CueO**	0.97 ± 0.88

SLAC and SLAC-VN were also purified using FPLC as described in section 2.1.1. Extremely low laccase activities (0.01 U/mg for SLAC; no activity detected for SLAC-VN) were observed after pooling the enzyme fractions collected by the fractionator. This may be attributed to the loss of copper at the enzyme's active site when exposed to the high pressure of the FPLC system, further supported by the lack of the characteristic blue colour for the small laccases. In an attempt to restore the copper, the enzyme samples were incubated overnight with 1 mM $CuSO_4$ at 4°C and dialysed against 10 mM potassium phosphate buffer to remove the excess unbound copper. A small increase in activity (0.05 U/mg for SLAC; 0.08 U/mg for SLAC-VN) was observed after the addition of copper and it was therefore decided to continue the purification of the bacterial laccases using the conventional size-exclusion chromatography. The loss in copper is most likely due to the high pressure of the FPLC interfering with the bonds and protein-protein interactions that are responsible for maintaining the tightly packed structure of SLAC. Similar phenomena are not reported in literature, unless copper depletion was intentionally performed using chelating agents.

3.2 BPA removal – Free laccases

3.2.1 pH optimisation

The effect of pH on the removal of BPA using free laccase was initially investigated at four pH values: pH 3.0, 5.0, 7.0 and 9.0. The experiments were performed at 22°C for 16 hours. Both fungal laccases optimally removed BPA at pH 5.0 (Figure 3.2). SLAC and SLAC-VN optimally removed BPA at pH 9.0 and pH 7.0, respectively. The pH optima was further refined at 0.5 pH value increments for each laccase (Figure 3.3).

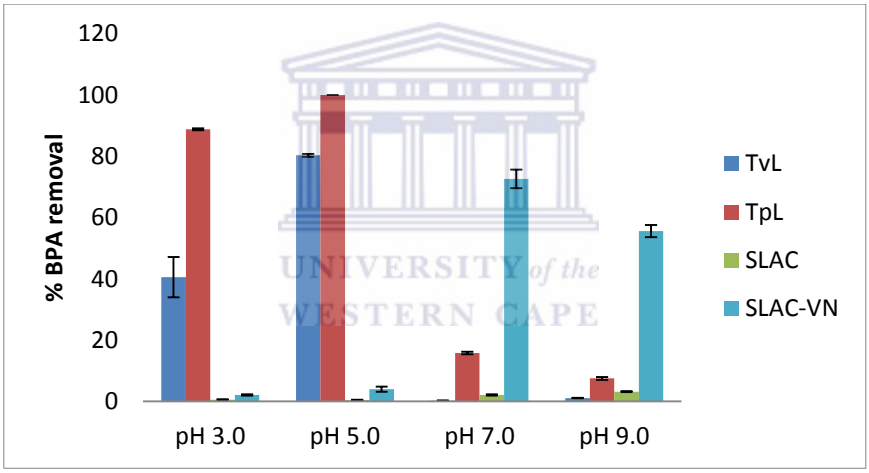

Figure 3.2: The effect of pH on the removal of BPA by laccases, TvL, TpL, SLAC and SLAC-VN. % removal of BPA as determined by HPLC represented as the mean of two experiments (mean ± standard deviation)

For TvL (Figure 3.3A), optimal BPA removal was observed at pH 5.5, pH 4.0 and 4.5 for TpL (Figure 3.3B), pH 9.5 for SLAC (Figure 3.3B) and pH 7.0 (Figure 3.3D) for SLAC-VN. The highest removal of BPA within 16 hours was observed for the fungal laccases (87% for TvL and 100% for TpL). SLAC was the least efficient, removing only 27% of BPA after incubation for 16 hours. Interestingly, the SLAC mutant, SLAC-VN, was able to remove BPA at a comparable level (93%) to that of the fungal laccases, but at a neutral pH.

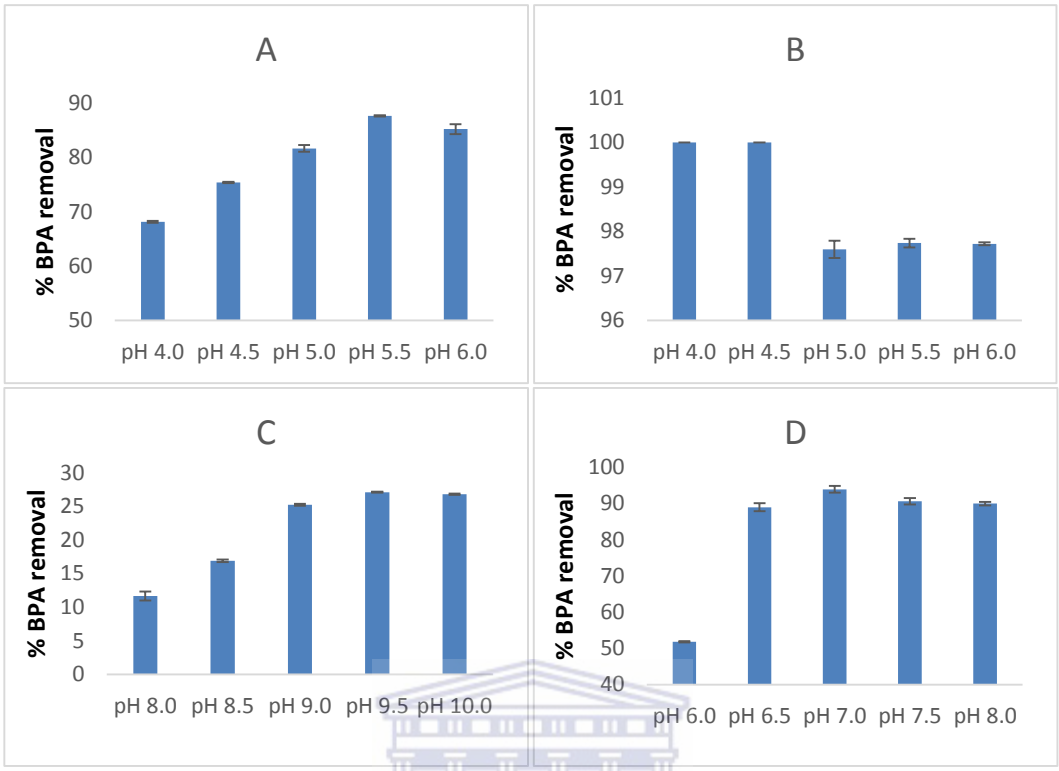

Figure 3.3: The effect of pH on the removal of BPA. pH conditions was further optimised by 0.5 pH value increments from the starting point as pre-determined per Figure 3.2 (A – TvL; B – TpL; C – SLAC; D – SLAC-VN). % BPA removal was determined by HPLC analyses.

Few studies report the effect of pH on the removal of BPA by free laccase. Kim and Nicell (2006) reported an optimal pH of 5.0 for the removal of BPA using a commercial preparation of laccase from *T. versicolor* (also purchased from Sigma-Aldrich), whilst a small difference was observed in our study where optimal removal of BPA occurred at pH 5.5 for *T. versicolor*. Similarly, Margot *et al.* (2013) compared the degradation of BPA, in addition to diclofenac and mefenamic acid, using TvL and the laccase from *Streptomyces cyaneus*. Whilst both laccases were able to significantly oxidise the micropollutants tested, the fungal TvL exhibited a higher oxidation rate at neutral pH when compared to the bacterial laccase from *S. cyaneus* (Margot *et al.,* 2013). In contrast, in our study, the bacterial SLAC-VN removed more BPA than its fungal

counterparts at neutral pH. It is to be noted, however, that both fungal laccases used in this study optimally removed the phenol-like BPA in the acidic range, as is common for fungal laccases (Baldrian *et al.,* 2006). Bacterial laccases, in contrast, function optimally at alkaline pH (Reiss *et al.,* 2011), as can be observed for SLAC. As a result to the introduced mutation of SLAC-VN had previously been shown to optimally oxidise phenolic-type compounds at neutral pH, as a result of the mutation (Prins *et al.,* 2015), which was also observed for the optimal removal of BPA at pH 7.0.

3.2.2 Temperature optimisation

Under optimal pH conditions, for each laccase, the removal of BPA was carried out at 20°C, 30°C and 40°C for 16 hours. For both fungal laccases, as well as for SLAC, optimal removal of BPA was observed at 30°C (Figure 3.4). For SLAC-VN, the highest percentage removal of BPA was observed at 40°C.

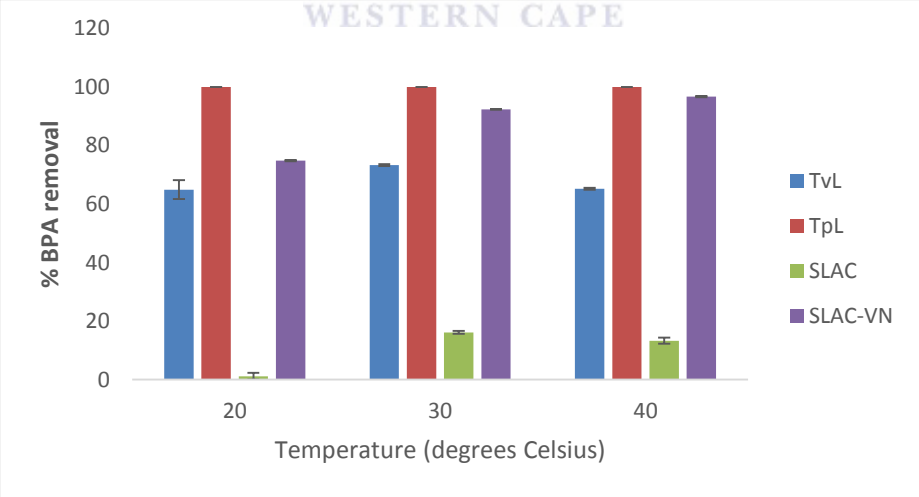

Figure 3.4: The effect of temperature on the removal of BPA. Duplicate experiments were performed at 20°C, 30°C and 40°C. Results are expressed as averages (mean ± standard deviation). % BPA was determined using HPLC analyses.

Kim and Nicell (2006) showed that laccase from *T. versicolor* optimally removed BPA at a temperature of 45°C, after which it begins to decline. In contrast, TvL used in this

study functioned optimally at 30°C and a decrease in the removal of BPA was observed when the temperature was increased to 40°C. This is interesting to note as the laccases used in both studies were commercial preparations purchased from Sigma-Aldrich. The exact reason for the decline in BPA removal is unknown. Kim and Nicell (2006) suggested that with an increase in temperature, two competing phenomena occur 1) an increase in the rate of thermal deactivation for the enzyme is observed, and 2) an increase in the rate of reaction is observed. Their results suggest that at temperature higher than 45°C, an increase in the rate of thermal deactivation cannot be compensated for by an increase in the rate of reaction, causing the degree of substrate conversion achieved in a one hour period to decline. Temperatures above 40°C were not used in this study, however, and it is therefore difficult to speculate whether this same effect is occurring. Furthermore, to the best of our knowledge, only one instance of an isolated bacterial laccase used for the removal of BPA has been described (Telke *et al.,* 2009) and the effect of temperature on the degradation of BPA was not reported in that study.

3.2.3 Optimisation of reaction times

The effect of incubation time on the removal of BPA was performed under optimal pH and temperature conditions. Reaction mixtures were incubated for 2, 8 and 16 hours. For TvL, TpL and SLAC-VN, more than 60% of BPA was removed within 2 hours of incubation (Figure 3.5). Within 16 hours, TpL outperformed all other laccases used in this study, however the bacterial laccase SLAC-VN was able to remove nearly 93% of BPA within 8 hours of incubation. This was three hours longer than the laccase from *Pseudomonas* sp. LCB1 (Telke *et al.,* 2009).

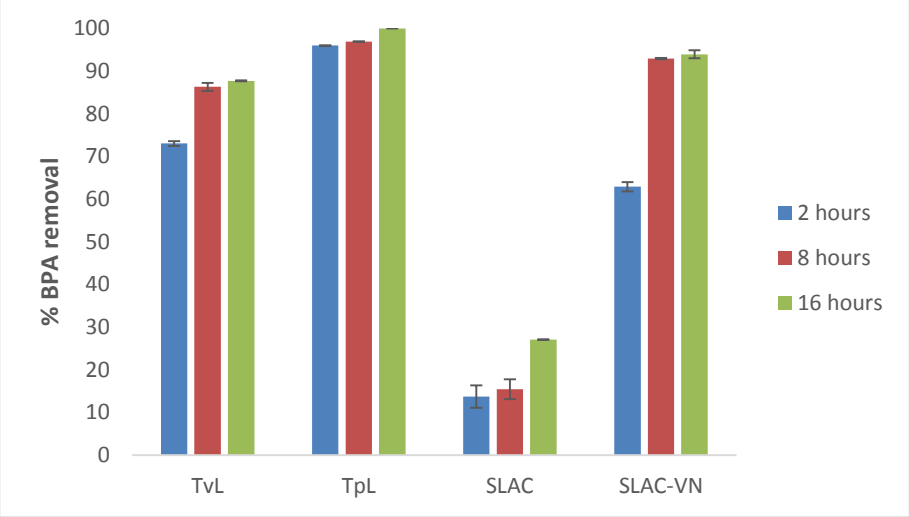

Figure 3.5: The effect of incubation time (2, 8 and 16 hours) on the removal of BPA (mean ± standard deviation). % BPA removal was determined by HPLC analyses.

3.2.4 BPA removal in the presence of redox mediators

Laccases are known to oxidise a wide number of compounds, without the aid of auxiliary compounds, such as mediators. In such cases, where the oxidation of certain compounds is not observed, the inclusion of a mediator compound may prove advantageous. Known laccase mediators were tested in order to determine their effectiveness at further promoting the removal of BPA. Two synthetic and two natural laccase mediators were tested at three different concentrations: 0.1 mM, 0.2 mM and 0.5 mM. In the presence of 0.5 mM 1-HBT, BPA removal was enhanced by 20% for TvL, compared to the percentage removal when mediators were omitted (Figure 3.6). For TpL, complete removal was observed in the presence of 0.5 mM vanillin, 0.5 mM ferulic acid and 0.1 mM 1-HBT. However, it should be noted that the effect of the mediators, in this case, was most likely negligible, especially since complete removal had previously been observed without the inclusion of any mediators (Figures 3.4 and 3.5) as well as in this experiment (Figure 3.6). For SLAC, a strong effect was observed; a nearly 6-fold increase BPA removal was observed for all four mediators, at all three

concentrations tested. It is interesting to note that with increasing amounts of mediator, an increase in the percentage removal was observed, albeit a small one. This did not, however, occur when TEMPO was used as mediator. When SLAC-VN was used, complete removal of BPA was observed in the presence of 0.2 mM and 0.5 mM vanillin.

The use of redox mediators to aid in the degradation of phenolic micropollutants has been widely investigated. Kim and Nicell (2006) demonstrated that the presence of 1-HBT and TEMPO had little to a slight negative impact on the degradation of BPA as compared to when no mediator was present. This agrees with our results where a negligible effect on the removal of BPA was observed for all laccase variants in the presence of 1-HBT and TEMPO, but especially for TvL at low concentrations of 1-HBT (0.1 mM and 0.2 mM) and at all concentrations of TEMPO that were tested. In contrast, the ability of the laccase from *C. polyzona* to degrade BPA was significantly enhanced by the inclusion of 0.2 mM 1-HBT (Cabana *et al*., 2007a).

While the use of phenolic mediators for the degradation of BPA has not been reviewed extensively, phenolic mediators have been applied to enhance the ability of laccase to degrade other phenolic micropollutants. For example, Kang *et al.* (2002) showed that the use of phenols substituted with one or two methoxy groups were highly effective at increasing the ability of the laccase from *Trametes villosa* to degrade the fungicide, cyprodinil. Johannes and Majcherczyk (2000) also demonstrated the use of two natural phenolic compounds, 4-hydroxybenzoic acid and 4-hydroxybenzylic alcohol for the degradation of polycyclic aromatic hydrocarbons (PAHs) by a fungal laccase.

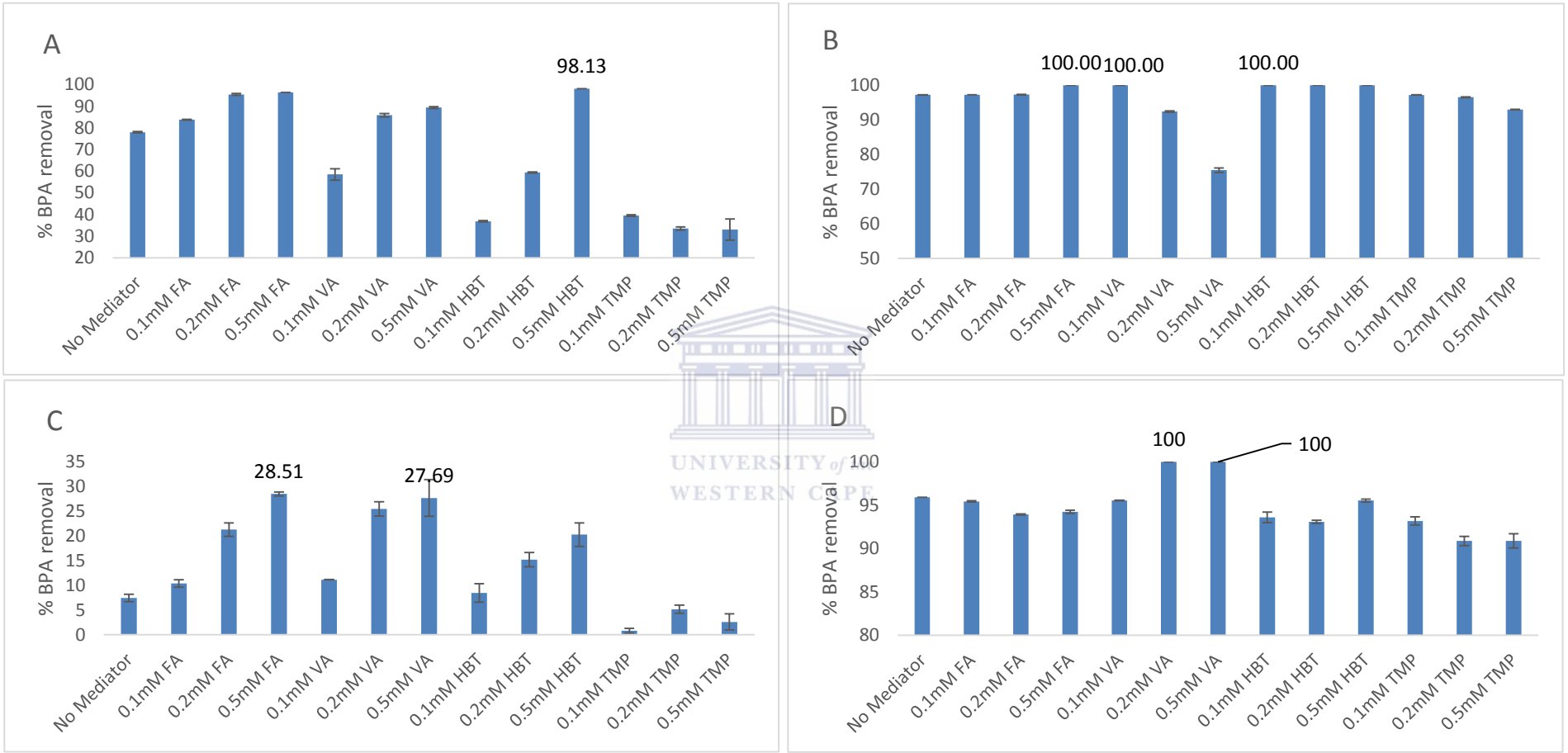

Figure 3.6: BPA removal performed under optimal pH and temperature conditions, in the presence of known laccase mediators, ferulic acid (FA), vanillin (VA), 1-hydroxybenzotriazole (1-HBT) and TEMPO (TMP) at varying concentrations (A – TvL; B – TpL; C – SLAC; D – SLAC-VN).

This demonstrates the advantages of using natural phenolic compounds, such as vanillin as a mediator for the laccase SLAC-VN for the removal of toxic compounds.

3.2.5 Putative metabolites

In order to identify which metabolites were formed by the laccases during the removal of BPA, samples that were prepared under optimal pH and temperature conditions were analysed by LC-MS. This was performed for each enzyme, both in the presence and absence of known laccase mediators. A metabolite profile was established for each enzyme, which consists of the predominant products that were identified from the samples' mass spectra (Table 3.2).

A number of putative metabolites were detected in this study (Appendix B). Predominant metabolites were cross-referenced with known compounds from literature and will be further elaborated on here. Compounds with mass-charge ratios of 453 m/z, 679 m/z and 905 m/z are indicative of a BPA dimer, trimer and tetramer, respectively (Figure 3.7). Compounds with mass-charge ratios of 94 and 133 are most likely phenol and 4-isopropenylphenol, respectively (Uchida *et al.,* 2001; Fukuda *et al.,* 2004).

Table 3.2: Compounds [represented by mass charge ratio (m/z)] identified for each enzyme variant in the presence and absence of known laccase mediators. Analysis was performed in negative mode – molecular weights indicated (-1 H^+). (Key: **-** not detected; **+** detected). The grey shading indicates oligomers of BPA, and intermediate products.

m/z	94	133	165	166	200	267	275	453	679	905
TvL	-	-	+	-	+	+	-	+	+	-
TpL	-	+	-	-	-	-	-	+	+	--
SLAC	+	+	+	-	+	+	-	+	+	-
SLAC-VN	+	+	-	-	+	+	-	+	+	-
TvL 0.5 mM HBT	+	+	-	-	-	-	-	+	-	-
TvL 0.5 mM FA	+	+	-	-	-	-	-	+	-	-
TpL 0.1 mM VA	-	+	-	-	-	-	-	+	-	-
TpL 0.1 mM HBT	-	+	-	-	-	-	-	-	-	-
SLAC 0.5 mM FA	-	-	-	-	-	-	-	-	-	-
SLAC 0.5 mM VA	-	-	-	-	-	-	-	+	+	-
SLAC-VN 0.5 mM VA	-	-	-	-	-	-	-	-	-	-

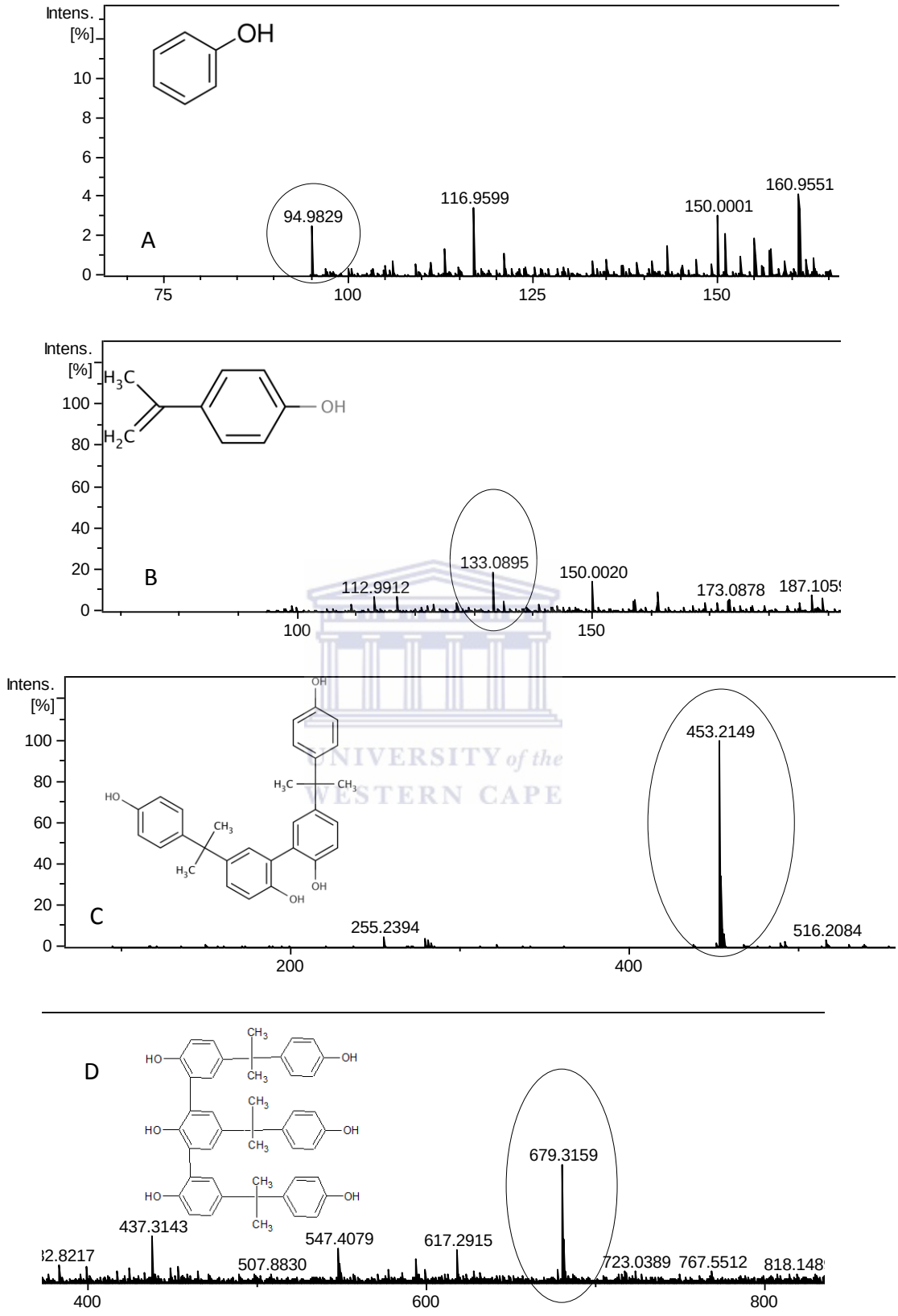

Figure 3.7: Mass spectra representative of major compounds (molecular weight indicated as mass-charge ratio) detected for BPA samples with laccase (analysis was performed in negative mode). A – phenol; B – 4-isopropenylphenol; C – BPA dimer; D – BPA trimer.

Fukuda *et al.* (2004) proposed a pathway for the degradation of BPA (Figure 3.8), which consists of two main processes: the first is the oxidative condensation of BPA to form a number of oligomers (as seen for all enzyme variants in the absence of redox mediators – Table 3.2). This oligomerisation occurs through the formation of C-C or C-O bonds between phenol moieties (Fukuda *et al.,* 2004). The presence of both phenol and 4-isopropenylphenol is a result of the second process, which entails the degradation of BPA dimers to yield 4-isopropenylphenol, leaving behind phenol moieties (Uchida *et al.,* 2001). Similar products were reported by Cabana *et al.* (2007a) when BPA was treated with laccase from *Coriolopsis polyzona*. Cabana *et al.* (2007a) also found that the BPA treated sample lost all of its oestrogenic activity after 8 hours of treatment with *C. polyzona* laccase. Similarly, Fukuda *et al.* (2004) failed to detect any oestrogenic activity from BPA oligomers in laccase treated samples, even when the supernatant contained large concentrations of these oligomers. Interestingly, the metabolite profiles generated in the presence of redox mediators were quite different. The BPA dimer was only observed for TvL in the presence of 0.5 mM ferulic acid and 0.5 mM 1-HBT, for TpL in the presence of 0.1 mM vanillin and for SLAC in the presence of 0.5 mM vanillin (Table 3.2). No metabolites corresponding to the aforementioned ones were observed for the samples treated with SLAC-VN in the presence of 0.5 mM vanillin. This may suggest the complete degradation of any BPA dimers that were formed and the further oxidation of 4-isopropenylphenol and phenol moieties by the laccase (Fukuda *et al.,* 2004).

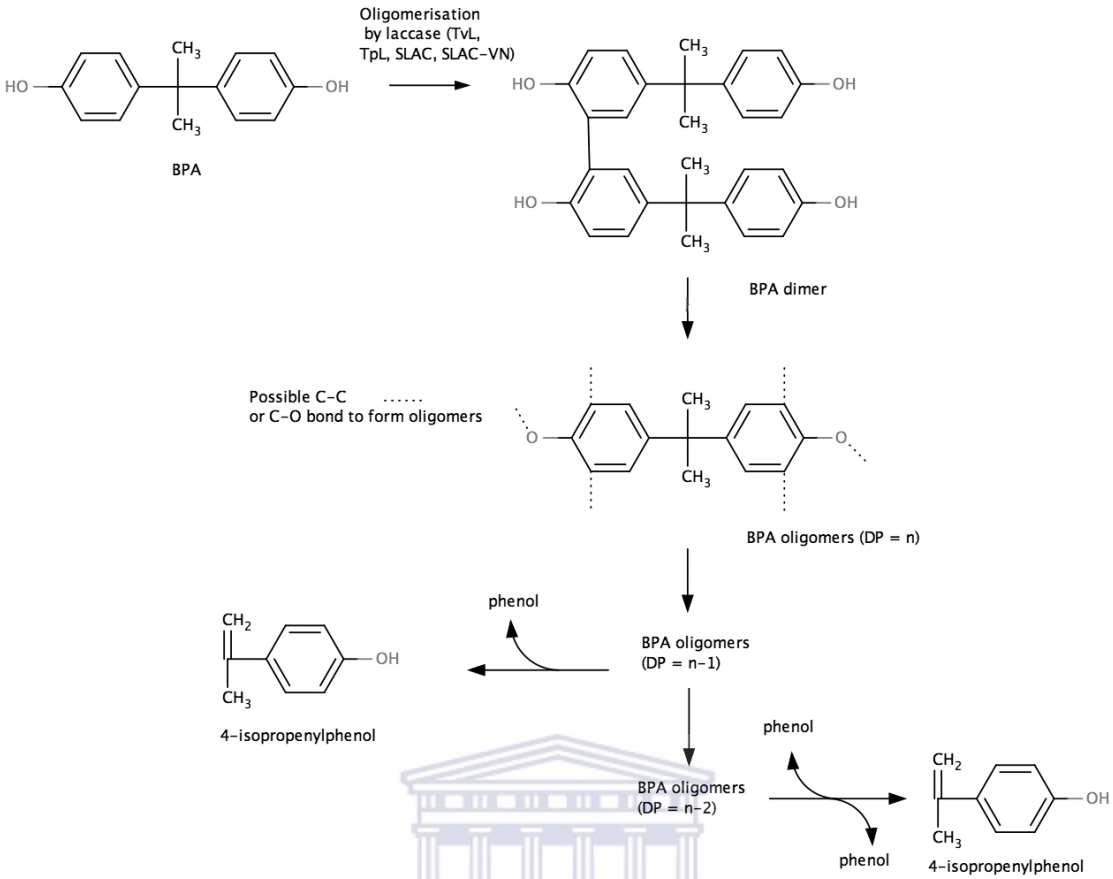

Figure 3.8: Proposed pathway for the degradation of BPA by laccases through a process of oligomerisation of BPA and subsequent degradation of BPA oligomers (adapted from Fukuda *et al.,* 2004)

3.3 Enzyme Immobilisation

Three immobilization strategies (encapsulation into alginate beads, and the production of CLEAs and CLECs) were investigated to determine the effect of the immobilisation on the ability of the enzymes to degrade BPA.

3.3.1 Alginate Bead Encapsulation

The laccases used in this study was encapsulated using varying concentrations of sodium alginate (1-4%). For each laccase, the amount of unbound laccase was determined in order to define the optimal concentration of sodium alginate to use that would allow for the most efficient enzyme loading. For TvL, the least amount of unbound laccase was observed when 3% sodium alginate was used to perform the encapsulation (Figure 3.9). For TpL, 2% sodium alginate was selected and 4% sodium alginate was selected for both bacterial laccases.

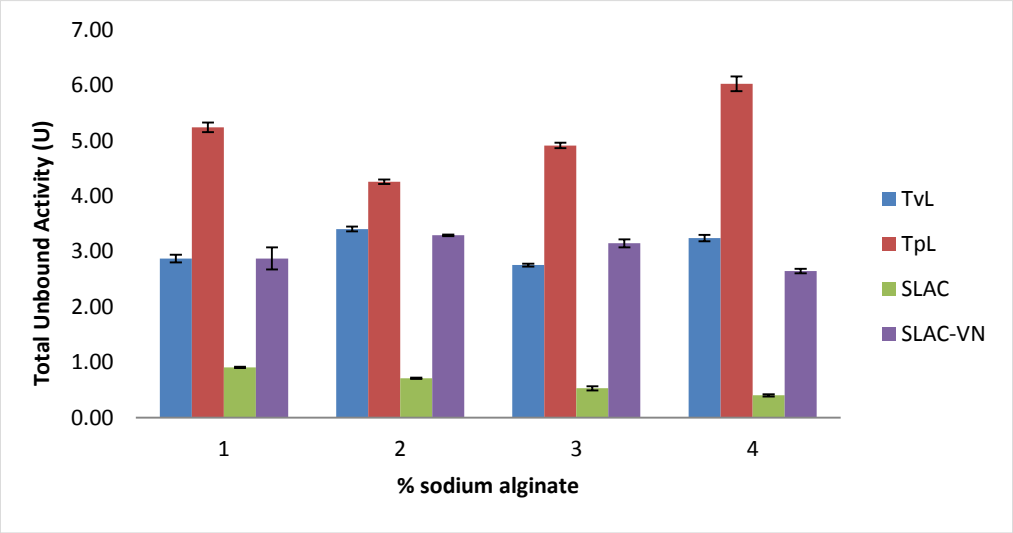

Figure 3.9: The amount of unbound laccase (U) was used to determine the optimal amount of sodium alginate required to allow for the best enzyme loading for each of the laccases used in this study.

Once the optimal sodium alginate concentration was selected, the percentage loading efficiency was calculated based on the amount of laccase added to each reaction versus the amount of unbound laccase that was measured. For TvL and TpL, the loading efficiency was determined to be 80% and 54%, respectively (Figure 3.10). For SLAC and SLAC-VN, a higher loading efficiency was observed (95% for SLAC; 82% for SLAC-VN). This may be attributed to the smaller size of the bacterial laccases, SLAC and SLAC-VN when compared to the larger fungal laccases. Similar values were reported by Palmieri *et al.* (1994) where up to 85% of activity was retained after entrapment of laccase from *Pleurotus ostreatus* into alginate beads, although in their case, copper was used as the cross-linking agent instead of calcium.

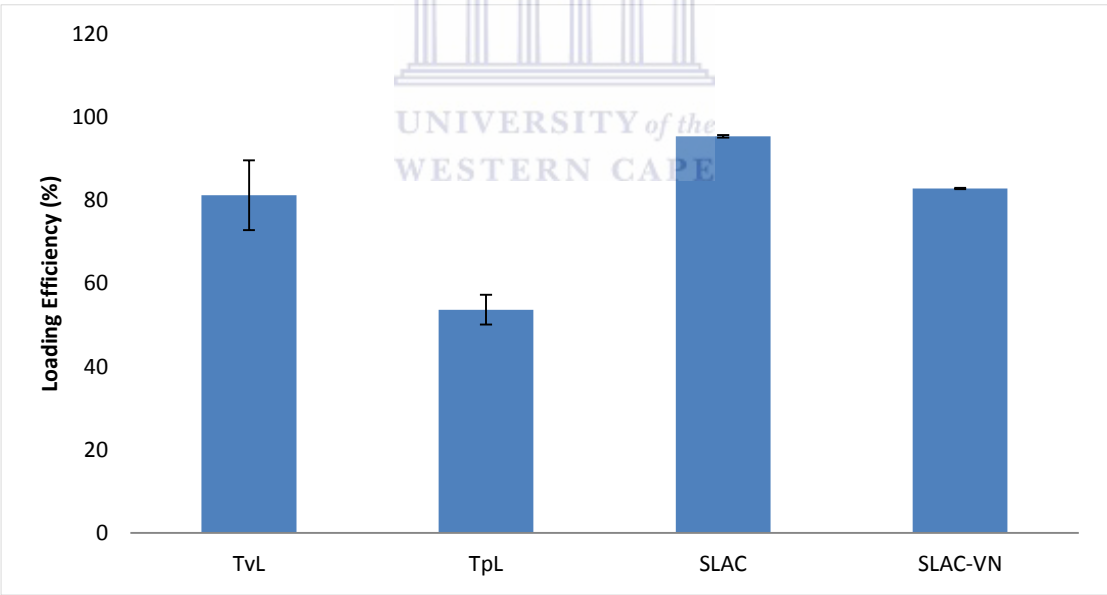

Figure 3.10: The percentage loading efficiency for fungal laccases (TvL and TpL) and bacterial laccases (SLAC and SLAC-VN)

Niladevi and Prema (2008) reported up to 42% loading efficiency when using calcium as the cross-linking agent for the encapsulation of laccase from *Streptomyces psammoticus*, while using copper yielded a loading efficiency of 62%.

3.3.2 Cross-linked Enzyme Crystals (CLECs)

A second immobilisation method was carried out by producing cross-linked enzyme crystals. This method entails the slow precipitation and crystallisation of the desired protein followed by the cross-linking of the crystals using a bi-functional agent, such as glutaraldehyde. Three hours after the addition of ammonium sulphate to a final concentration of 75% and gentle stirring, all laccase samples became opaque indicating the presence of precipitated protein (Figure 3.11). However, when the samples were centrifuged in order to separate the "crystals" it was observed that the protein had formed large aggregates (not dissimilar to CLEAs).

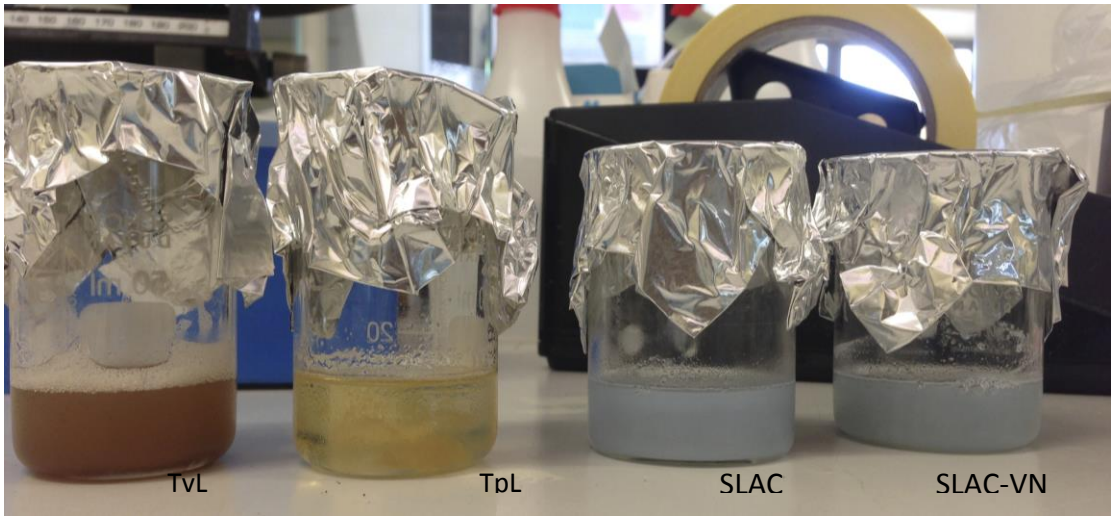

Figure 3.11: Laccase samples after the addition of ammonium sulphate to a final concentration of 75%. Large precipitant formed as indicated by opacity of the solution

Nonetheless, the supernatant was kept for analysis and the "crystals" were incubated with 1.5% glutaraldehyde to allow any cross-linking to occur. The production of the CLECs was unsuccessful. Except for TpL, for each laccase, the amount of unbound laccase nearly equalled the amount of laccase added to the reactions (Table 3.3). It is most likely that the cross-linking failed due to the inadequate formation of enzyme crystals.

Table 3.3: The loading efficiency for CLECs produced from fungal and bacterial laccases. In almost all cases, loading efficiency was 0% due to the high amount of unbound laccase activity.

	Laccase added (U)	Unbound laccase (U)	Loading efficiency (%)
TvL CLECs	4.26 ± 0.20	5.98 ± 0.78	0%
TpL CLECs	1.78 ± 0.21	1.66 ± 0.35	7%
SLAC CLECs	1.02 ± 0.02	1.08 ± 0.29	0%
SLAC-VN CLECs	1.46 ± 0.01	1.49 ± 0.20	0%

One major drawback of CLECs is the requirement to crystallise the enzyme, which is often a laborious process and laccase of an extremely high purity is required (Margolin, 1996; Sheldon and van Pelt, 2013). The lack of crystal formation for the laccases used in this study is most likely as a result of impurities in the sample. Excessive cross-linking may also have caused ineffective immobilisation as this can lead to aggregation (as observed for the laccase samples in this study) or a disturbance of the crystalline lattice (Govardhan, 1999). Sheldon and van Pelt (2013) outlined the prohibitively high costs of obtaining enzyme of an extremely high purity and thereby highlighted the trend towards the use of the closely related Cross-Linked Enzyme Aggregates (CLEAs).

3.3.3 Cross-linked Enzyme Aggregates (CLEAs)

The production of cross-linked enzyme aggregates was the third immobilisation strategy that was performed. TvL, TpL, SLAC and SLAC-VN were precipitated in the presence and absence of 50 mg BSA. The cross-linking of the laccase aggregates that were formed was performed using glutaraldehyde at concentrations of 1, 2 and 3%. A higher degree of cross-linking was observed for both fungal and bacterial laccases when co-aggregated with BSA (Figure 3.12).

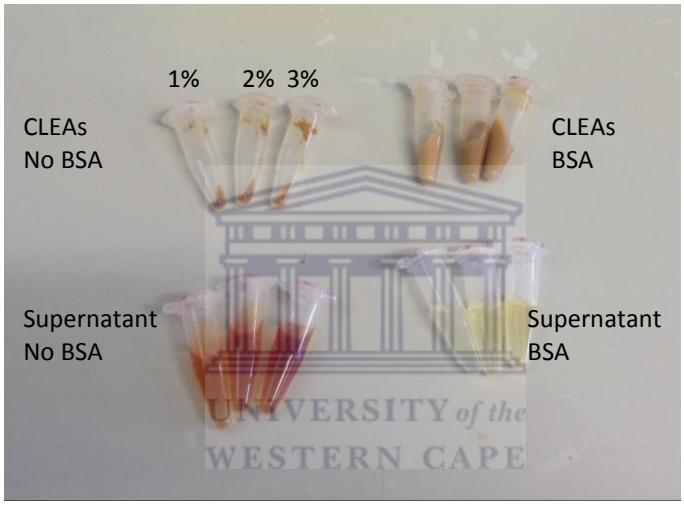

Figure 3.12: An example of the CLEAs produced from SLAC in the presence and absence of BSA. Cross-linking of the aggregates was performed using 1, 2 and 3% glutaraldehyde. A higher degree of cross-linking is observed in the presence of BSA, indicated by the lighter colour of the supernatant containing BSA.

For both the fungal laccases, TvL and TpL, the lowest residual laccase activity was observed when cross-linking was performed with 1% glutaraldehyde in the presence of 50 mg BSA, retaining approximately 50% of its initial activity (Figure 3.13sA).

For the bacterial laccases, 2% glutaraldehyde in combination with BSA was selected as the optimal conditions for CLEA production. A 2-fold increase in activity was observed for SLAC (0.21 U/mL compared to 0.12 U/mL), while the activity for SLAC-VN increased by four times the initial activity (Figure 3.13B). Cabana *et al.* (2007b), in

contrast, reports a decrease in the percentage recovery and a decrease in the laccase activity of the CLEAs formed from *C. polyzona* laccase – a similar trend is observed for the fungal laccases used in this study.

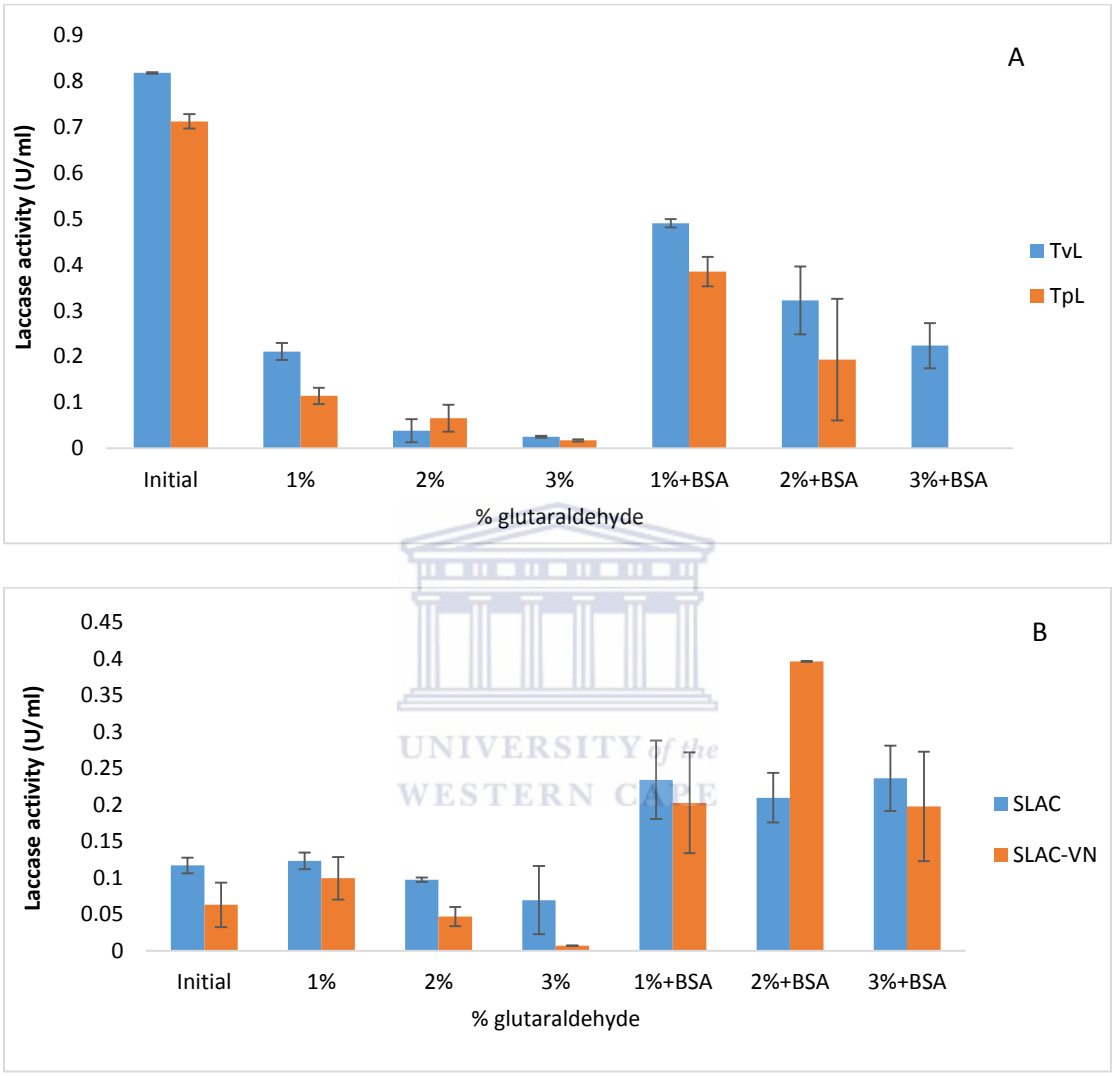

Figure 3.13: Activity (U/mL) of fungal (A) and bacterial (B) laccase CLEAs (50 µL dispersed) determined using 1 mM ABTS (pH 4.0) at 20˚C

The choice of precipitant used also seems to play a large role in the overall recovery of laccase activity when producing CLEAs. For example, Matijosyte *et al.* (2010) investigated the effect of three precipitants [dioxane, 2-propanol and 1,2-dimethoxyethane (DME)] on the percentage activity recovered when forming CLEAs from commercial *T. versicolor* laccase (Sigma). Only 22% of laccase activity was

recovered when using DME as a precipitant compared to more than 50% activity recovered in this study where ammonium sulphate was used as the precipitant.

3.4 BPA removal by encapsulated laccase

3.4.1 pH optimisation

The effect of pH on the removal of BPA using alginate-encapsulated laccase was initially investigated at four pH values: pH 3.0, 5.0, 7.0 and 9.0. The experiments were carried out at 22°C for 16 hours. TvL and TpL optimally removed BPA at pH 5.0 (Figure 3.14), the same initial pH as when the enzyme were tested in their free form. Optimal BPA removal occurred at pH 9.0 and pH 7.0 for SLAC and SLAC-VN, respectively. It is to be noted that when encapsulated, the overall removal of BPA by the laccases is lower than when the enzymes were not encapsulated.

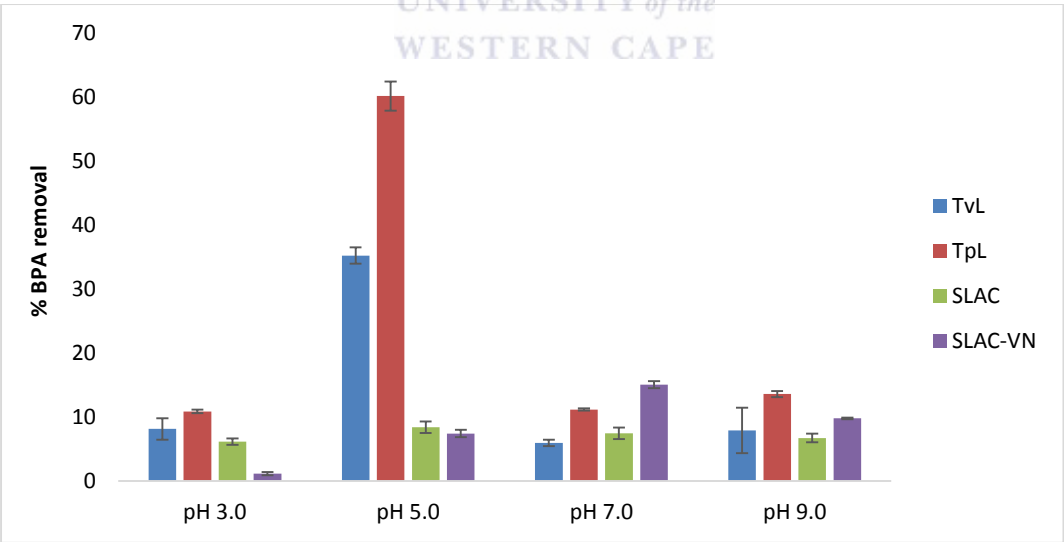

Figure 3.14: The effect of pH on the removal of BPA using alginate-encapsulated laccase TvL, TpL, SLAC and SLAC-VN. Initial pH optimisation performed at pH 3.0; 5.0; 7.0 and 9.0. % BPA removal was determined by HPLC analyses.

The pH optima were further optimised at one pH value above and below the previously determined values (Figure 3.15). Further optimisation of the optimal pH for TpL revealed a shift of one pH value upward (pH 6.0) (Figure 3.15B) compared to pH 5.0 when the enzyme is in its free form. The pH optima for the other laccases remained largely unchanged when compared to their free form (Figure 3.15A, C and D).

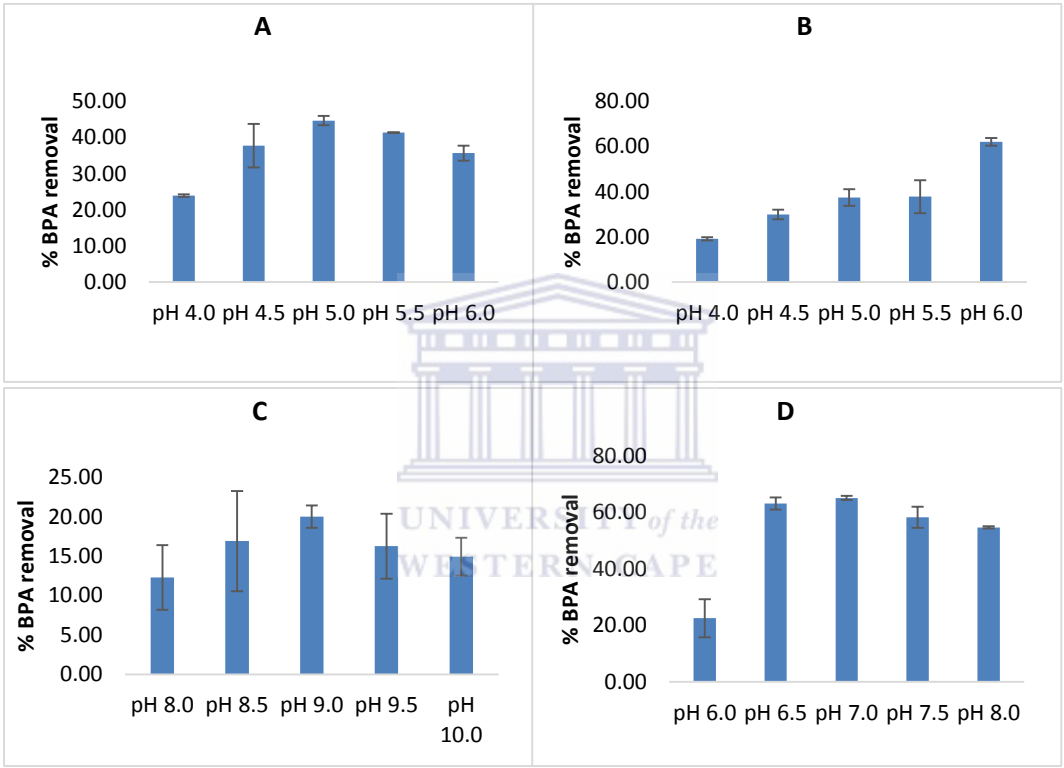

Figure 3.15: The effect of pH on the removal of BPA using alginate encapsulated laccase (A – TvL; B – TpL; C – SLAC; D – SLAC-VN). pH was further optimised at one pH value above and below the previously determined optimal pH. % BPA removal was determined by HPLC analyses.

The pH shift for TpL is not surprising as the immobilisation of laccases is often associated with improved characteristics such a broadened pH range (Kunamneni *et al.,* 2008). The decreased removal of BPA when using encapsulated laccase was also expected as this methodology of enzyme immobilisation is often characterised by mass transfer limitation (Brady and Jordaan, 2009; Fernandez-Fernandez *et al.*, 2013).

Once again, it is difficult to draw a direct comparison for the removal of endocrine disrupting chemical using encapsulated laccase due to a lack of published data. While the practice of encapsulating laccase within alginate beads is widely reported, many studies focus on the use of encapsulated laccases for application in the treatment of other environmental pollutants, such as dye decolourisation (Teerapatsakul *et al.,* 2008; Mogharabi *et al.,* 2012). The closest comparison is a study reported by Niladevi and Prema (2008) where they used a copper-alginate mixture to encapsulate laccase from *S. psammoticus* for the removal of phenol from a phenol model solution. Within 6 hours, phenol was reduced to 70%, along with 72% colour removal (Niladevi and Prema, 2008). This demonstrates the potential of alginate-encapsulated laccase for application in the removal of toxic micropollutants.

3.4.2 Temperature optimisation

The effect of temperature on the removal of BPA using alginate encapsulated enzyme was performed under the optimal pH for each enzyme. The experiments were carried out at 20, 30 and 40°C for 16 hours. Optimal BPA removal was observed at 40°C for both the fungal and bacterial laccases (Figure 3.16), with SLAC-VN exhibiting a slightly higher removal rate than either of the fungal laccases (59% BPA removal for SLAC-VN compared to 57% and 54% for TvL and TpL, respectively).

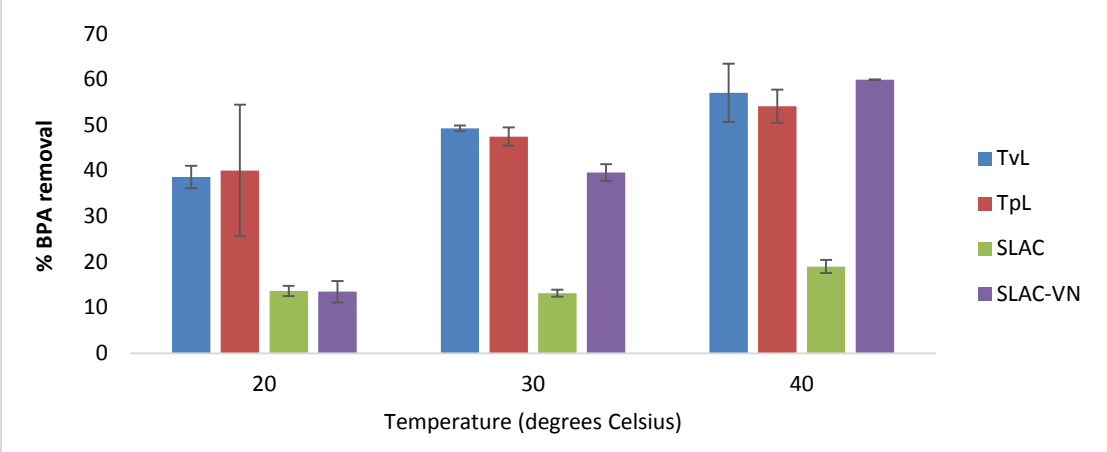

Figure 3.16: The effect of temperature on the removal of BPA using alginate-encapsulated laccase. Temperature optimisation was performed at 20, 30 and 40˚C. % BPA removal was determined by HPLC analyses.

3.4.3 Optimisation of reaction times

The effect of reaction times on the removal of BPA using alginate-encapsulated laccase was determined under optimal pH and temperature conditions. When free laccases were used, TvL, TpL and SLAC-VN were able to remove more than 60% BPA within two hours of incubation. When the encapsulated laccases are used, the limited rate of substrate and enzyme interaction as a consequence of encapsulation comes into play (Fernandez-Fernandez *et al.,* 2013). Less than 40% removal of BPA occurred within 8 hours of incubation, indicating the effect of encapsulation on the enzyme activities. It is interesting to note, however, that the bacterial laccase, SLAC-VN, was able to remove more BPA than the fungal laccase.

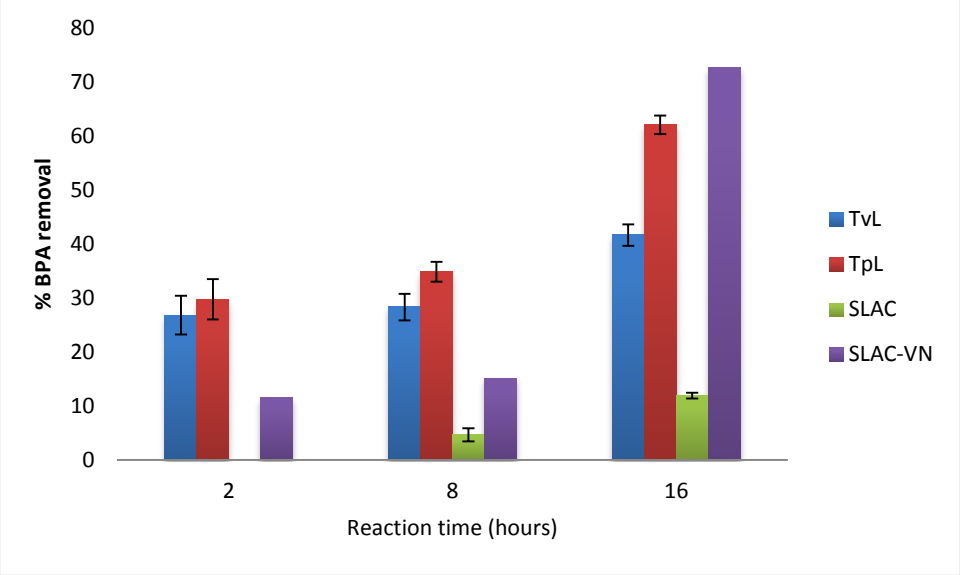

Figure 3.17: The effect of reaction times (in hours) on the removal of BPA using alginate encapsulated laccase. Reactions were incubated for 2, 8 and 16 hours prior to HPLC analyses.

3.4.4 BPA removal in the presence of redox mediators

Four known laccase mediators were used to determine whether they are able to enhance the BPA removal capability of the encapsulated laccases. For TvL, TpL and SLAC, at any given concentration, all the laccase mediators tested with the exception of TEMPO, an increase in the removal of BPA was observed with an increase in mediator concentration. For TvL, TpL and SLAC, the highest BPA removal was observed in the presence of 0.5 mM ferulic acid (Figure 3.18A, B and C). In fact, in the presence of 0.5 mM ferulic acid, the removal efficiency of the encapsulated fungal laccases was significantly enhanced and matched the removal efficiency of the fungal laccases in their free form. The highest BPA removal in the presence of a mediator for SLAC-VN was observed when 0.5 mM vanillin was used (Figure 3.18D), even though the percentage removal was lower than when no mediator was used (42% BPA removal compared to 51% removal in the absence of a mediator). SLAC-VN showed 72% BPA removal without the use of a mediator (Figure 3.17). However, it is to be noted that

this discrepancy may be linked to the amount of enzyme encapsulated in the beads. It is possible that beads used in the mediator systems contained less than the theoretically calculated amount of 0.5 U of laccase. Consequently, the removal of BPA in this case (51% in the absence of a mediator) was lower than when determining the effect of reaction time, even though the same amounts of beads were used to conduct the experiments.

Interestingly, a previous report suggested that the structure of copper-alginate might cause a restrictive environment to enzyme/mediator interactions and limits the diffusion of the radical species of the mediator to the surrounding environment (Brandi *et al.*, 2006). In this study, the opposite seems to hold true and may be attributed to the less rigid nature of calcium-alginate when compared to copper alginate.

3.4.5 Stability of encapsulated laccase

The stability of the encapsulated laccases was determined by incubating the beads under the optimal pH and temperature conditions for each laccase, for selected time periods (24, 48, 72, 96 and 120 hours) prior to conducting the BPA removal experiments with the beads. For both fungal and bacterial laccases, the BPA removal capability was dramatically affected. The alginate beads containing TvL was able to retain the most activity (34% BPA removal) after 24 hours of incubation when compared to the other laccases (Figure 3.19). TpL and SLAC-VN, both of which

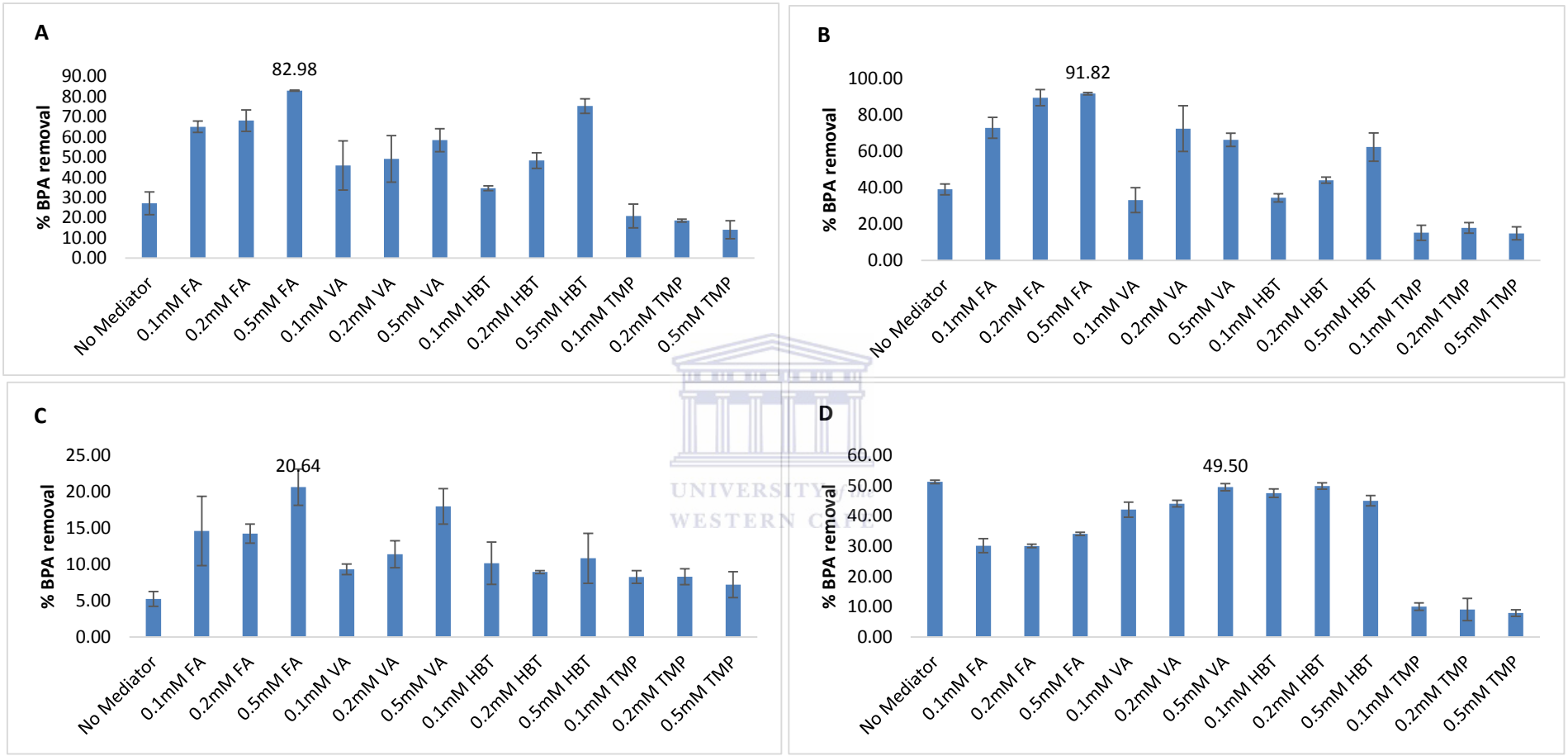

Figure 3.18: The removal of BPA in the presence of known laccase mediators using alginate-encapsulated laccase (A – TvL; B – TpL; C – SLAC; D – SLAC-VN). For each laccase variant, four known laccase mediators were tested at concentrations of 0.1 mM, 0.2 mM and 0.5 mM. % BPA removal was determined by HPLC analyses.

were capable of removing up to 60% BPA within 16 hours, were only able to remove 28% and 20% of BPA, respectively.

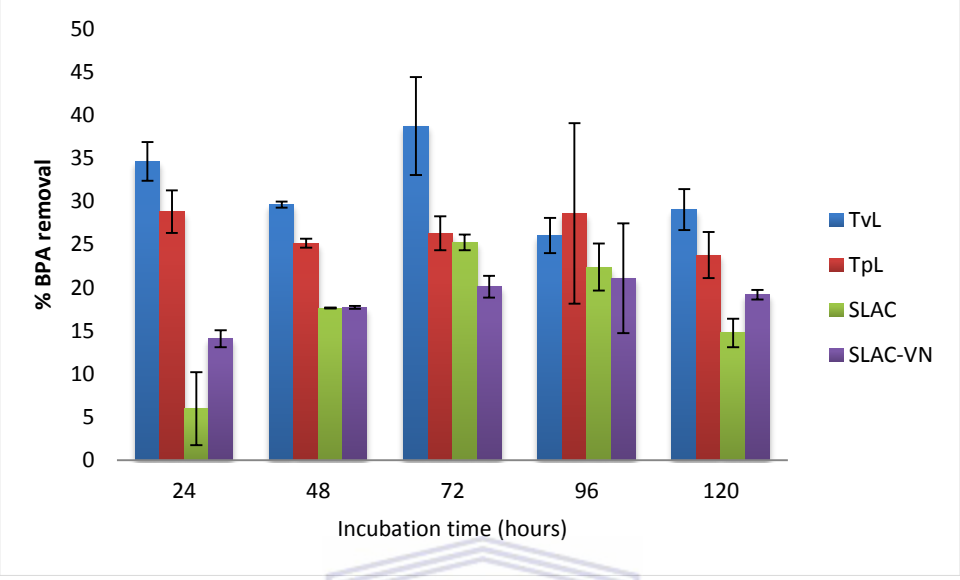

Figure 3.19: The effect of extended incubation times on the stability of alginate-encapsulated laccase. % BPA removal was determined after incubating the alginate beads at the optimal pH and temperature conditions for each enzyme for the allotted time periods (24 hours – 120 hours).

Previous studies have shown similar results: The loss in activity observed here is attributed to the lower chemical stability of calcium-alginate, which consequently leads to a higher rate of enzyme leakage from the beads (Brandi *et al.,* 2006). Using copper as the divalent cross-linking agent when forming the beads can counteract this instability, rather than the calcium used in this study in order to enhance the rigidity of the bead structure. This is not to say that the copper-alginate beads are completely exempt from bead removal. Palmieri *et al.* (1994) demonstrated that prolonged incubation of copper alginate beads containing laccase from *P. ostreatus* at 37°C led to the continuous release of enzyme from the beads, which is likely due to the repeated swelling and relaxing of the gel matrix.

3.4.6 Reusability of encapsulated laccase

The aim of any immobilisation method is to enable the continuous use of an enzyme for a number of consecutive runs, while maintaining the majority of the enzyme's activity. The encapsulated laccases used in this study were reused under optimal pH and temperature conditions for a total of five cycles. Each cycle lasted for 16 hours. The maximum BPA removal capability was lost for all enzymes after just one cycle. During the second cycle, BPA removal dropped to less than 30% for all the enzymes tested (Figure 3.20). This is most likely due to the low chemical stability of the beads when undergoing prolonged exposure to elevated temperatures, as explained in section 3.4.5. What was interesting to note was that by the fourth cycle, the beads for TvL, TpL and SLAC started to disintegrate and a fifth cycle could not be performed for these enzyme. It is unknown why the beads for SLAC-VN were able to stay intact for five cycles, albeit with a relatively low BPA removal efficiency of just 14%.

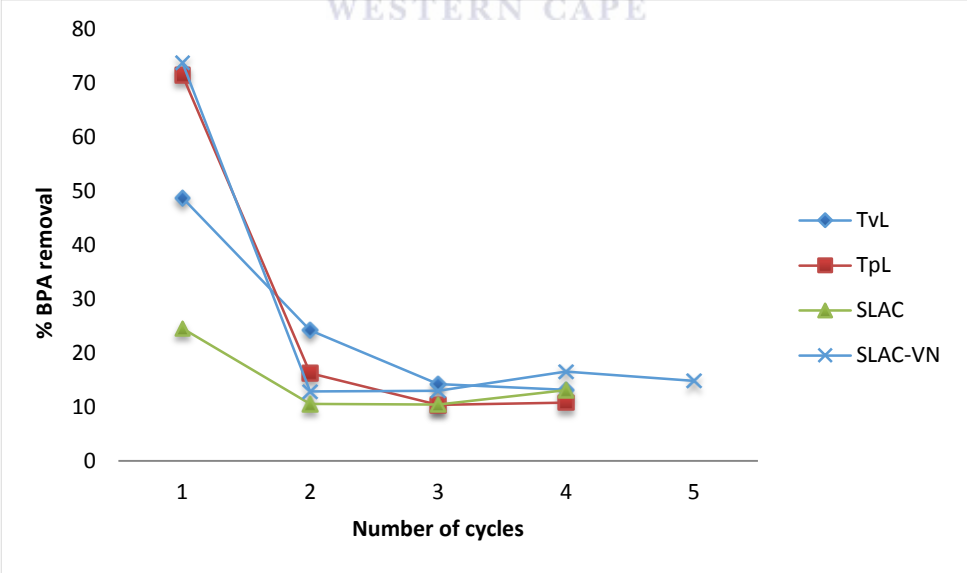

Figure 3.20: The reusability of alginate-encapsulated laccase under optimal conditions through a number of cycles. Each cycle lasted 16 hours, after which the beads were washed and added to a fresh reaction mixture containing 10 mg/L BPA. % BPA was determined by HPLC analyses.

Brandi *et al.* (2006) reports the recycling of laccase from *Trametes villosa* encapsulated in both copper- and calcium-alginate beads. Most of the activity is lost

(20% relative activity) after three cycles of use (24 hours per cycle) when calcium-alginate beads were used, compared to nearly double that when copper-alginate beads were used. This once again demonstrates the robustness of copper as a cross-linking agent for use in the formation of alginate beads, and that alginate beads formed using calcium as a cross-linker are not suitable for the treatment of BPA.

3.4.7 Putative metabolites

Once the optimal pH and temperature conditions for the removal of BPA using alginate-encapsulated laccase were known for each of the laccase variants, BPA removal experiments were performed under these conditions and analysed by LC-MS (Appendix B). The putative metabolite profiles differed quite a bit as when compared to the free enzyme was used. In the absence of mediators, the dimer of BPA was only detected for the samples treated by TpL. The BPA dimer, trimer and, what is most likely to be the BPA tetramer, were detected in the samples treated by SLAC-VN (Figure 3.21). For the samples treated with TvL, a compound with a mass charge ratio of 134 m/z (4-isopropenylphenol) was observed, suggesting that some degree of polymerisation had occurred. No known metabolites were observed for SLAC.

In the presence of redox mediators, 4-isopropenylphenol was detected only for TpL-treated samples. The reason some of the products were not detected may be attributed to the limitation in the diffusion of compounds across the bead barrier, when alginate beads are used (Brandi *et al.,* 2006). It's very likely that the metabolites

are trapped inside and would require the complete degradation of the beads to release any compounds of interest into the surrounding supernatant.

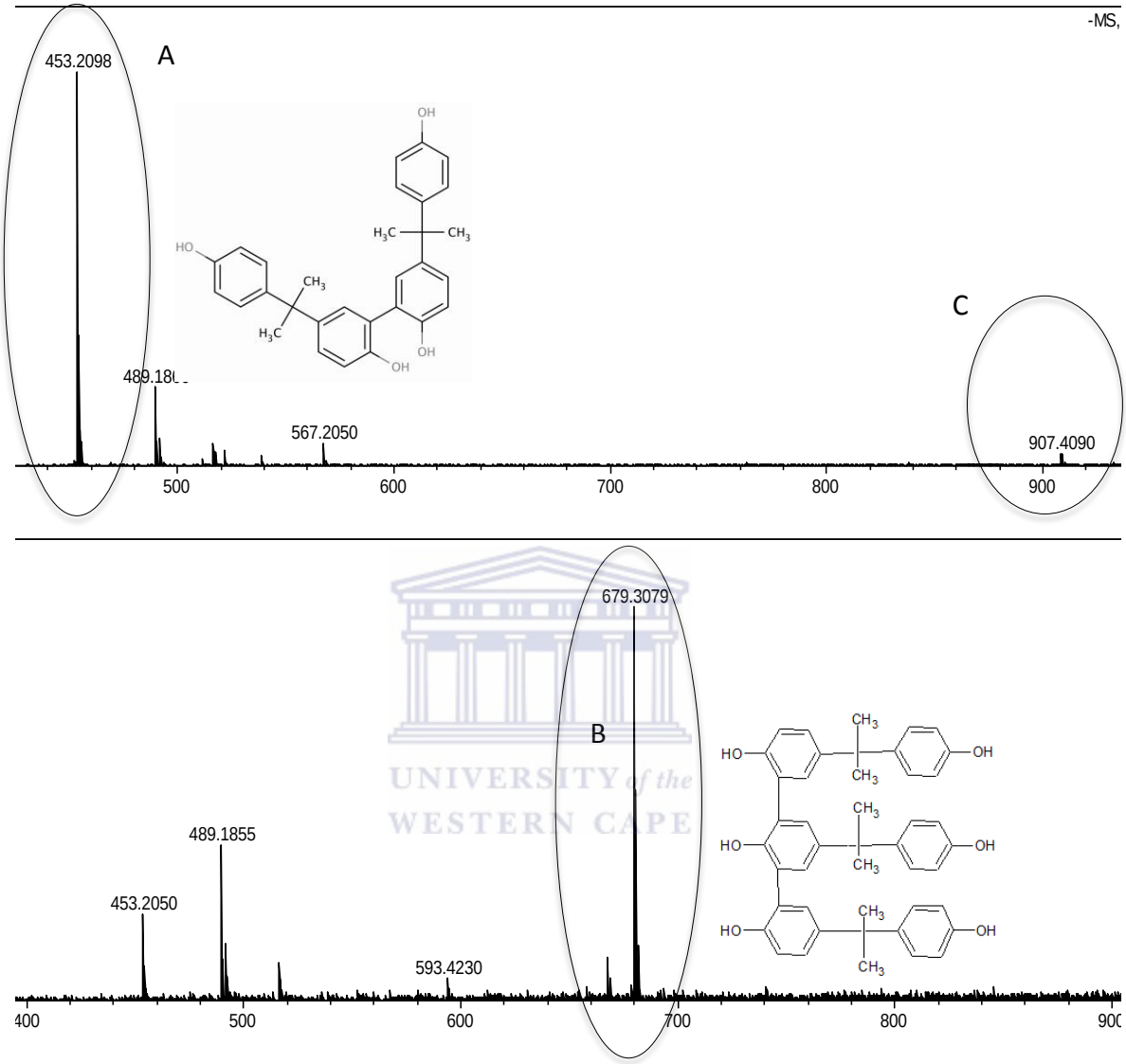

Figure 3.21: Mass spectra representing the major BPA oligomers formed when treating BPA with SLAC-VN in the absence of mediators. Mass spectra was collected in negative mode. A – BPA dimer (453 m/z), B – BPA trimer (679 m/z); C – BPA tetramer (907 m/z).

3.5 BPA removal by laccase CLEAs

The third immobilisation strategy tested was the use of cross-linked enzyme aggregates for the removal of BPA.

3.5.1 pH optimisation

The effect of pH on the removal of BPA using laccase CLEAs was investigated at four pH values initially: pH 3.0, 5.0, 7.0 and 9.0. These experiments were performed at 22°C. The fungal laccase, TvL, was able to remove 96% of BPA at pH 5.0 within 16 hours (Figure 3.21). Two notable changes are observed with regards to pH profile and BPA removal for TvL, TpL and SLAC as CLEAs. TpL optimally removed BPA at pH 9.0, compared to pH 5.0 in its free form. This is a dramatic shift in pH optima as fungal laccases tend to function optimally at acidic pH. For TvL, the same extension of pH range was observed, although the optimum remained at pH 5.0. In CLEA form, SLAC removed BPA at a much higher rate than in its free form: Up to 70% removal was observed, compared to less than 30% when SLAC was in its free form or encapsulated in sodium alginate. These changes can be attributed to the increased stability and specificity introduced when the CLEAs were produced (Cabana *et al.,* 2007b).

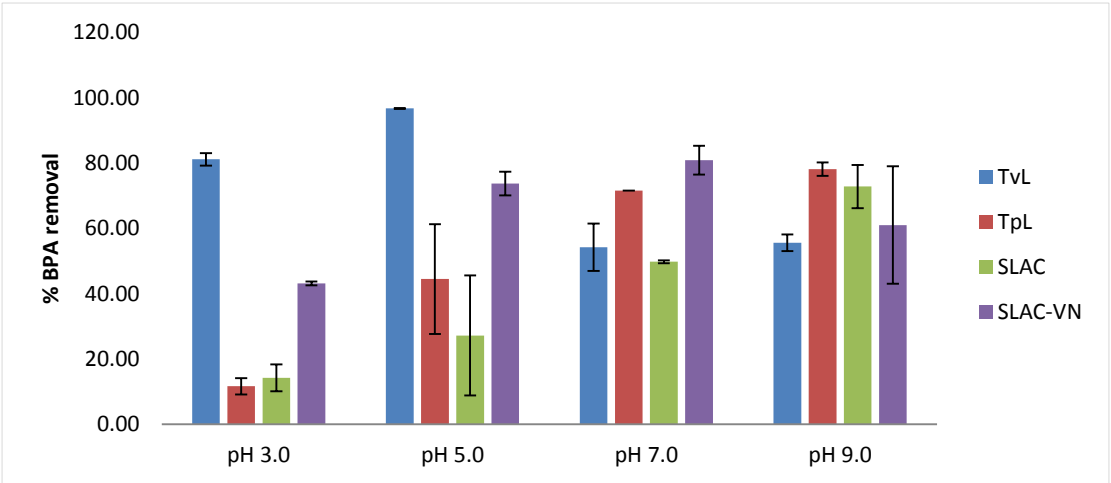

Figure 3.22: The effect of pH on the removal of BPA using laccase CLEAs produced from TvL, TpL, SLAC and SLAC-VN. pH optimisation was initially performed at pH 3.0; 5.0; 7.0 and 9.0. % BPA removal was determined using HPLC analyses.

The pH optima were further optimised at one pH value above and below the previously determined range. TvL optimally removes BPA across a range of pH values (pH 4.5-6.0) (Figure 3.22A). The mid-point, pH 5.0, was therefore selected for further studies. The other laccase CLEAs showed more distinctive pH profiles. TpL optimally removed BPA at pH 8.5, whilst SLAC and SLAC-VN optimally removed BPA at pH 8.0 and 7.0, respectively (Figure 3.22A and B).

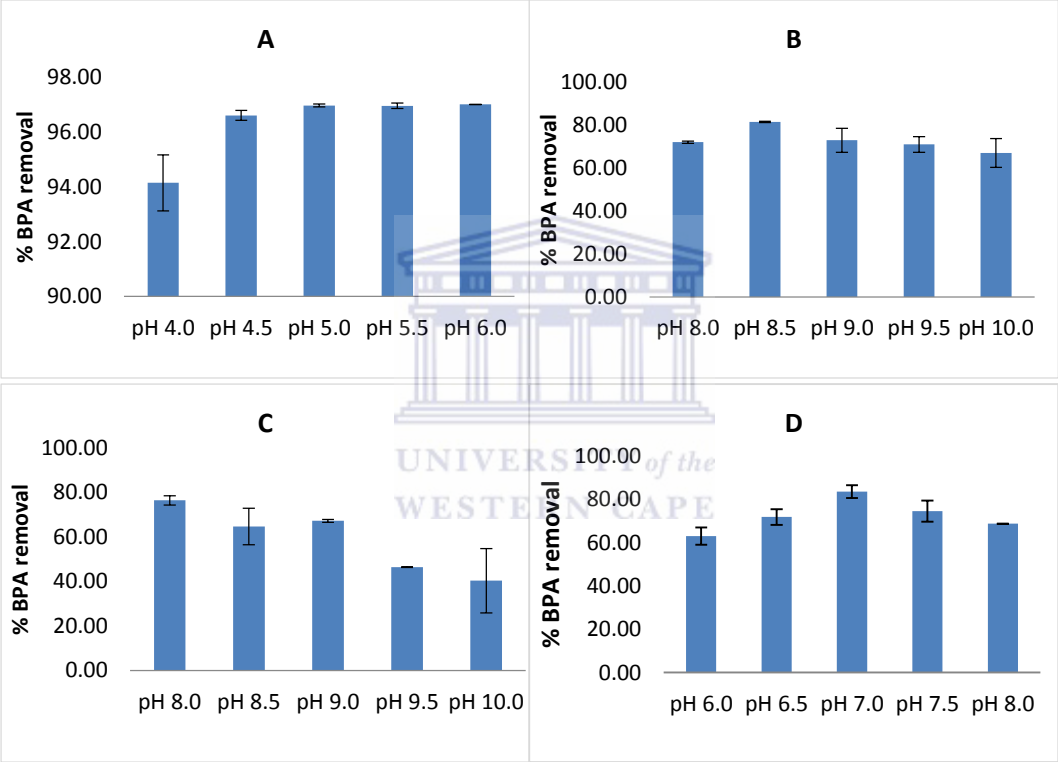

Figure 3.23: The effect of pH on the removal of BPA using laccase CLEAs (A – TvL; B – TpL; C- SLAC; D – SLAC-VN). pH optimisation was further optimised at one pH value above and below the previously determined value. % BPA removal was determined using HPLC analyses

3.5.2 Temperature optimisation

The optimal temperature for the removal of BPA was determined under optimal pH conditions for each laccase. The reactions were incubated at 20˚C, 30˚ and 40˚C for 16 hours. The CLEAs made from TvL was the most robust, maintaining 96% BPA removal at all temperatures tested. TpL and SLAC functioned optimally at 20˚C and the highest

activity for SLAC-VN was observed at 30˚C. These temperatures are lower than that observed when comparing these enzyme to their optimal operating temperatures when the free enzymes were used, while still maintaining a high degree of BPA removal. This would make them more suited for application in an industrial process where energy input in terms of heating large quantities of wastewater would be an impractical.

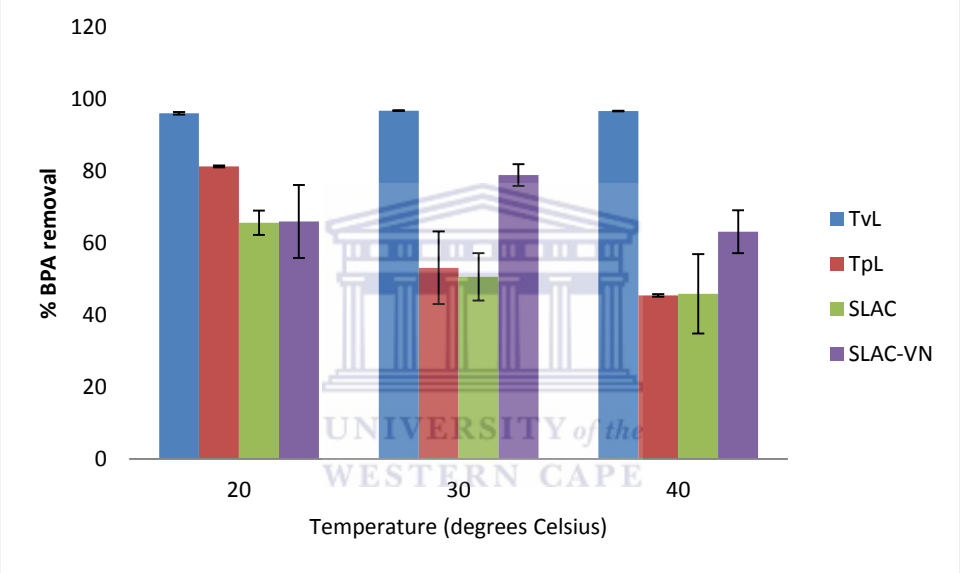

Figure 3.24: The effect of temperature on the removal of BPA using laccase CLEAs. Temperature optimisation was performed at 20, 30 and 40˚C. % BPA removal was determined by HPLC analyses.

To date, only Cabana *et al.* (2007b) has reported the use of laccase CLEAs for the degradation of BPA. The CLEAs were produced in the presence and absence of BSA. Whilst they tested the use of ammonium sulphate as a precipitant, the laccase from *C. polyzona* was most effectively precipitated using 100% polyethylene glycol. *C. polyzona* CLEAs was able to degrade more than 93% of BPA (5 mg/L) when it was used in a fluidised bed reactor. Not only were the CLEAs effective at removing BPA, they were also resistant to chemical denaturants and the CLEAs formed in the presence of BSA were especially thermostable (Cabana *et al.,* 2007b).

In this study, the CLEAs were also tested for stability. After 16 hours, the supernatants of the samples that were incubated at 20°C, 30°C and 40°C, were analysed for laccase activity, as well as analysed by SDS-PAGE to determine whether any laccase leached out. The amount of laccase detected in the supernatant was highest for TvL, with a maximum of 0.28 U/mL detected for the sample exposed to 30°C (Figure 3.25). This suggests that the high degree of BPA removal observed for the TvL CLEAs, are as a result of a combination of CLEAs and free enzyme that leached from the CLEAs.

The higher amount of laccase activity detected for TvL in the supernatant may be as a result of the higher specific activity (U/mg) that is typical of this fungal laccase, compared to TpL. This is further supported by the lack of enzyme subunits visible on the SDS-PAGE gel analysis of the post-reaction supernatants (Figure 3.26). The only band visible on the gel, is that of BSA, which may be leaching from the CLEA because it is in excess (Figure 3.26A and 3.27A).

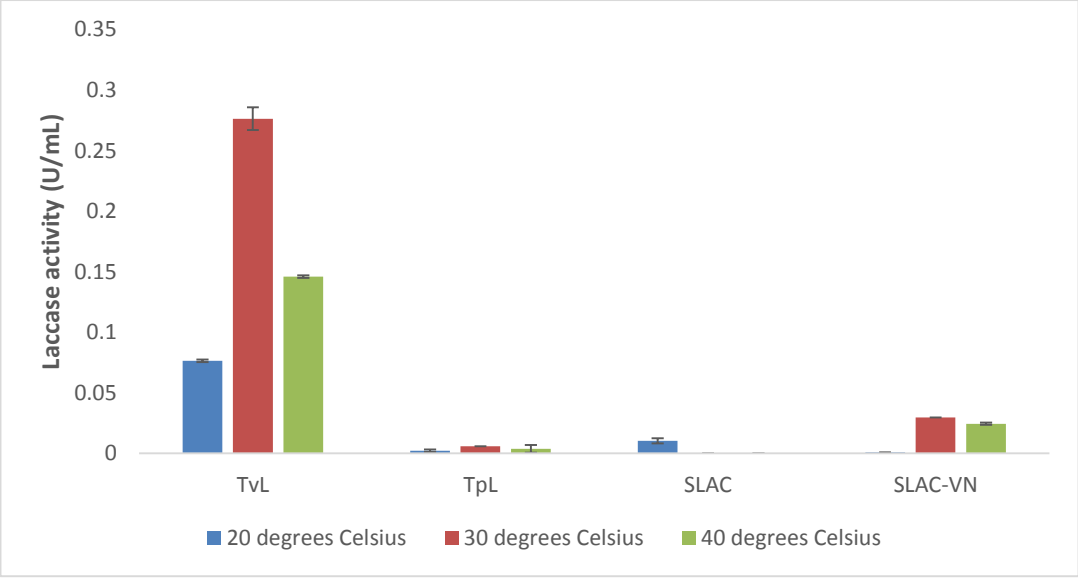

Figure 3.25: Laccase activity (U/mL) measured in the supernatant of BPA removal reactions using laccase CLEAs after 16 hours incubated at 20°C, 30°C and 40°C.

Furthermore, with the exception of TvL, the presence of high molecular weight aggregates that were unable to enter the gel for all enzymes tested, suggests that the CLEAs remained largely intact (Figure 3.26B and Figure 3.27B). The high level of BPA removal and the potential enhancements these CLEAs present, warrants further investigation (stability when exposed to prolonged temperature, reusability) in a future study.

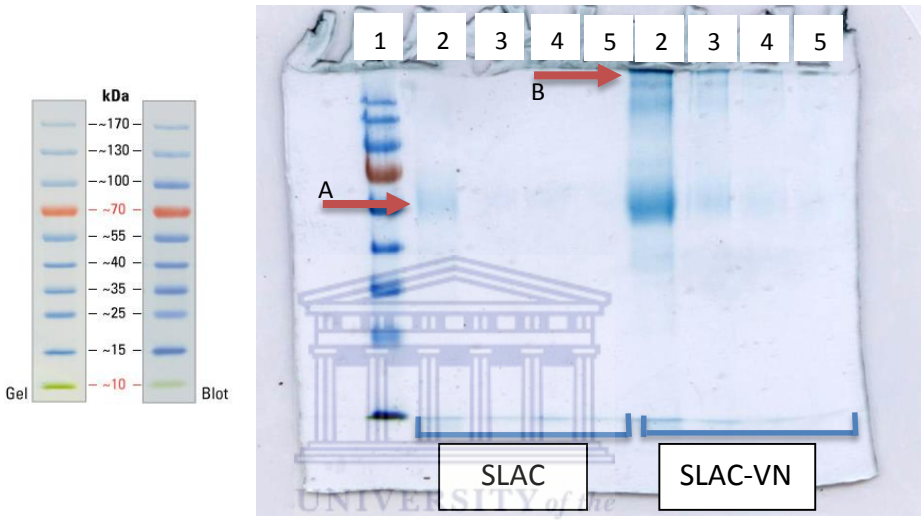

Figure 3.26: SDS-PAGE of bacterial laccase CLEAs. Molecular weight marker in Lane 1. Untreated CLEAs in Lane 2. Samples were supernatants from BPA removal experiments after 16 hours incubation at 20°C [3], 30°C [4] and 40°C [5]. BSA (A) observed at approximately 66 kDa and high molecular weight CLEAs observed at stacking/resolving gel interface (B).

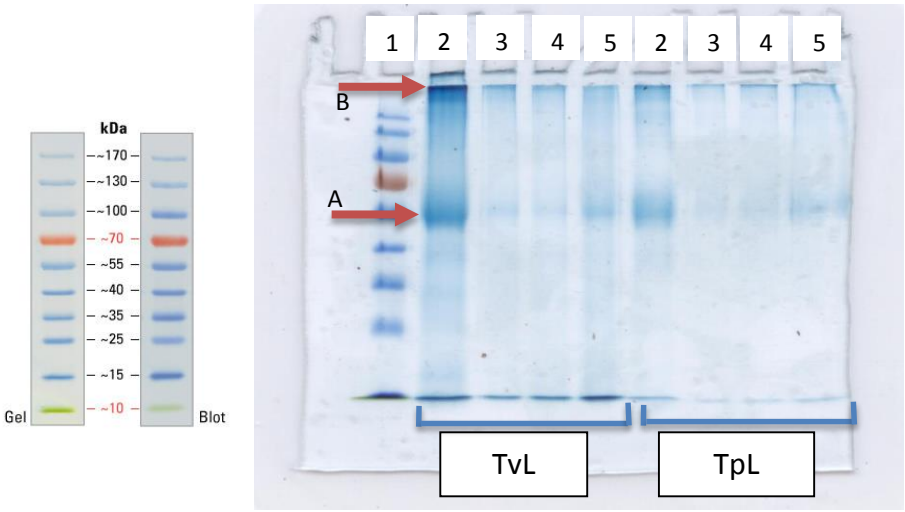

Figure 3.27: SDS-PAGE of fungal laccase CLEAs. Molecular weight marker in Lane 1. Untreated CLEAs in Lane 2. Samples were supernatants from BPA removal experiments after 16 hours incubation at 20°C [3], 30°C [4] and 40°C [5]. BSA (A) observed at approximately 66 kDa and high molecular weight CLEAs observed at stacking/resolving gel interface (B).

3.5.3 Putative metabolites

Under optimal pH and temperature conditions for the laccase CLEAs, the mass spectra of the samples were taken. Poor ionisation of the samples was observed, for all laccases tested (Figure 3.28A), especially when compared to a sample that gave good signals (Figure 3.28B). The reason behind the poor ionisation may be as a result of low quantities of the analytes in the sample. No BPA metabolites, including the predominant BPA dimer, trimer or tetramer that were previously referenced from literature, were detected for any of the samples. From the few peaks that were distinct from the baseline, no known metabolites were detected, either. It is likely that the reason none of the main known metabolites were detected, is that the BPA was completely mineralised to CO_2 and water – this will be confirmed in future studies.

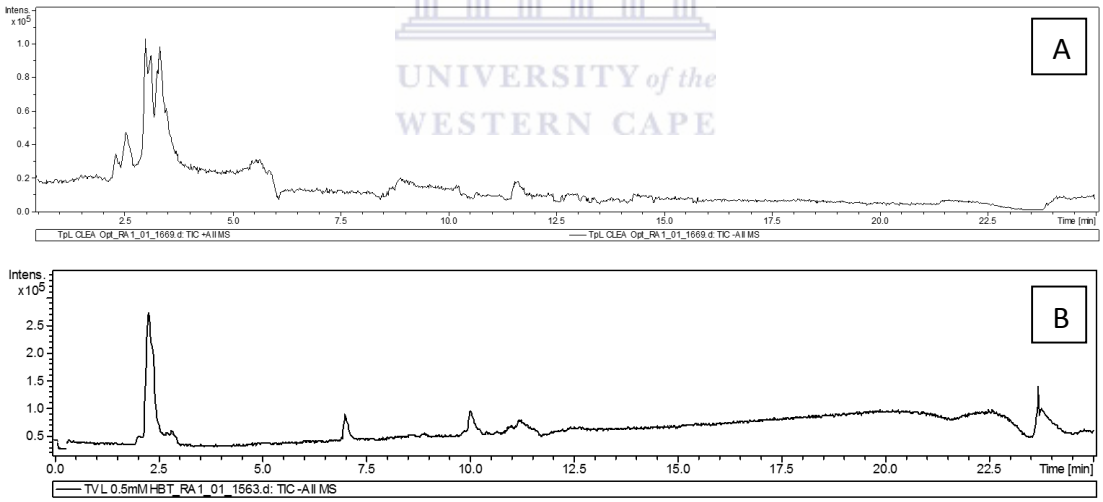

Figure 3.28: Chromatogram of BPA sample treated with TpL CLEAs (A) and free enzyme in the presence of 0.5 mM 1-HBT (B). Poor ionisation (low peak intensity, noisy baseline) of sample in A may attribute to poor detection of putative BPA metabolites. Note the well-defined peaks in chromatogram B.

CHAPTER FOUR

SUMMARY AND CONCLUDING REMARKS

The degradation of the endocrine disrupting chemical, bisphenol A (BPA), is one of high importance and research efforts are continually being focused on this topic. In this study, the ability of two fungal laccases (TvL and TpL) and two bacterial laccases (SLAC and SLAC-VN) to degrade BPA were compared, the major findings of which are summarised in Table 4.1. The removal of BPA was tested while the enzymes were in their free form, encapsulated in calcium alginate beads and self-immobilised in cross-linked enzyme aggregate (CLEA) form.

In their free form, the highest removal of BPA was exhibited by the fungal laccase, TpL (100% removal) and the bacterial mutant laccase SLAC-VN (96% removal). This result is remarkable for a bacterial laccase, which is typically outperformed by the higher redox potential fungal laccases. All laccases functioned optimally at 30°C, with the exception of SLAC-VN, which exhibited the most efficient removal of BPA at 40°C. In terms of the removal of BPA and putative metabolites, LC-MS analyses showed that all laccases tested were able to oligomerise BPA into dimers, trimers and tetramers of BPA. All laccases were also able to further degrade BPA, as evidenced by the presence of two intermediates, 4-isopropenylphenol and phenol, that are released upon the further degradation of BPA oligomers.

Table 4.1: The effect of the different immobilisation techniques on the ability of the laccases tested to degrade BPA. Major products produced were analysed by LC-MS analyses.

	Optimal pH	Optimal temp (°C)	% BPA removal	Reusable (cycles)	Stability (hrs. until activity loss)	Major putative metabolites (m/z)
TvL free	5.5	30	73	n.d.	n.d.	453 (dimer), 679 (trimer)
TvL encaps.	5.0	40	57	4	24	134 (4-isopropenylphenol)
TvL CLEA	5.0	20	96	n.d.	n.d.	None detected
TpL free	4.5	30	100	n.d.	n.d.	134, 453, 679
TpL encaps.	6.0	40	54	4	24	453
TpL CLEA	8.5	20	96	n.d.	n.d.	None detected
SLAC free	9.5	30	16	n.d.	n.d.	94 (phenol), 134, 453, 679
SLAC encaps.	9.0	40	19	4	24	None detected
SLAC CLEA	8.0	20	65	n.d.	n.d.	None detected
SLAC-VN free	7.0	40	96	n.d.	n.d.	94, 134, 453, 679
SLAC-VN encaps.	7.0	40	59	5	24	453, 679, 907 (tetramer)
SLAC-VN CLEA	7.0	30	78	n.d.	n.d.	None detected

**n.d. – not determined

Two immobilisation strategies were tested, the first of which was the encapsulation of the laccases in sodium alginate beads using calcium as the cross-linking ion. The pH range of the laccases was largely unaffected, with the fungal laccases still optimally removing BPA in the acidic range (pH 5.0 for TvL and pH 4.5 for TpL). The bacterial laccase, SLAC, exhibited the highest BPA removal in the alkaline range, albeit very low removal. Interestingly, the SLAC mutant, SLAC-VN, was able to remove BPA at a slightly higher rate than the fungal laccases (59% for SLAC-VN compared to 54% for TpL and 57% for TvL). The encapsulation of the laccases in alginate beads also extended the optimal temperature for the removal of BPA, with all laccases now able to function at 40°C. The putative metabolite profiles generated by the laccases were slightly different for all laccases, except for SLAC-VN, which was still oligomerising BPA into dimers, trimers and tetramers. The lack of putative metabolites for TvL, TpL and SLAC may be attributed to the limited rate of diffusion across the barrier of the alginate bead, causing any potential metabolite to remain inside the bead and not diffuse into the surrounding environment.

The final immobilisation strategy performed was the self-immobilisation technique of producing CLEAs. Interestingly, this method of immobilisation had the most significant effect on the characteristics of the laccases used in this study. Firstly, the pH range for both fungal laccases was greatly extended beyond the acidic range, with TpL optimally degrading BPA at pH 8.5 [compared to previous pH values of 6.0 (encapsulated) and 4.5 (free)]. Even though the optimal pH for BPA removal was pH 5.0 for the TvL CLEAs, TvL was able to remove BPA across a pH range of 3-9. Even more remarkable, the formation of SLAC CLEAs greatly enhanced the bacterial laccase's ability to remove

BPA. Previously, the maximum percentage removal of BPA by SLAC was approximately 25% with the aid of a redox mediator. In CLEA form, however, SLAC was able to remove approximately 60% of BPA within 16 hours of incubation. No known putative metabolites were detected by LC-MS analyses when CLEAs were used for the removal of BPA. While the possibility exists that the CLEAs were able to completely oxidise BPA (to CO_2 and water), confirmation tests are required to concentrate the samples and reanalyse for putative metabolites.

The effect of four known laccase mediators [two synthetic (1-HBT and TEMPO) and two natural (ferulic acid and vanillin)] on the ability of the laccases to remove BPA was also determined. The effect of the mediators on the laccases' ability to remove BPA was negligible at best, with the exception of SLAC. An approximate 6-fold increase BPA removal was observed when SLAC was used in its free form for the removal of BPA in the free of ferulic acid and vanillin (up to concentrations of 0.5 mM). The same effect on BPA removal was observed when encapsulated SLAC was used in the presence of ferulic acid and vanillin. The results suggest that SLAC may be even more efficient at BPA removal, when used in CLEA form, in the presence of the two natural laccase mediators.

Future Studies and Research Outputs

Due to time constraints, the complete optimisation for the laccase CLEAs was not finalised. Future work will include determining the optimal reaction times, stability of the CLEAs under optimal pH and temperature conditions for an extended period of time, the effect of redox mediators on the removal of BPA using CLEAs and whether

the CLEAs are suitable for continuous use while maintaining an efficient level of BPA removal.

Apart from the known compounds, a variety of other metabolites may exist in the sample mixtures, as well as potential products that may be trapped in the alginate beads. These metabolites may have oestrogenic properties, but this can only be confirmed by additional tests that screen for oestrogenic activity. Future work for this study includes the extraction and purification of the putative metabolites for each of the immobilisation approaches using Solid Phase Extraction (SPE) and testing the putative metabolites for oestrogenic activity. The oestrogenic activity tests will be performed at the Department of Urology, University of Pretoria under the supervision of Dr. Natalie Aneck-Hahn. A manuscript for publication of this work is currently being planned.

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APPENDIX A

A1: *Trametes* Defined Medium (TDM)

In 2 L distilled H_2O, the following was combined:

20.0 g glucose

10.46 g peptone

4.0 g KH_2PO_4

1.0 g $MgSO_4.7H_2O$

0.2 g $CaCl_2.2H_2O$

0.58 g NaCl

20 mL TDM trace salts

(500 mL 100X TDM trace salts: 0.28 g iron sulphate, 0.016 g copper sulphate, 0.034 g zinc chloride, 0.169 g manganese, 0.095 g cobalt chloride, 0.0012 g nickel chloride, 0.309 g ammonium molybdate)

A2: 2x YT medium

In 800 mL dH_2O, the following was combined:

16.0 g tryptone

5.0 g yeast extract

5.0 g NaCl

The pH was adjusted to 7.0 before making up to final volume of 1 L.

A3: Buffers

***100 mM sodium acetate (pH 5.0)* – 1 L**

- 0.2 M acetic acid was prepared by mixing 11.55 mL glacial acetic acid with 500 mL dH_2O and adjusting the volume to 1 L. 0.2 M sodium acetate solution was prepared by dissolving 27.2 g of sodium acetate trihydrate in 800 mL of water and making up the volume to 1L. 100 mM sodium acetate (pH 5.0) was prepared by combining 148 mL of acetic acid (0.2 M), 352 mL of sodium acetate solution (0.2 M) and 500 mL of dH_2O.

***100 mM potassium phosphate (pH 7.3)* – 1 L**

- 0.2 M KH_2PO_4 solution was prepared by dissolving 27.2 g of KH_2PO_4 in 500 mL dH_2O and adjusting the volume to 1 L. 0.2 M K_2HPO_4 solution was prepared by dissolving 34.8 g of K_2HPO_4 in 500 mL of water and making up the volume to 1L. 100 mM potassium phosphate buffer (pH 7.3) was prepared by combining 115 mL of KH_2PO_4 (0.2 M), 385 mL of K_2HPO_4 (0.2 M) and 500 mL of dH_2O.

***50 mM ammonium bicarbonate (pH 6.0 – 10.0)* – 100 mL**

- In 80mL dH_2O, 0.395 g of ammonium bicarbonate was dissolved. The pH was adjusted to desired value using glacial acetic acid (decrease) or ammonia (increase) before making up to final volume of 100 mL.

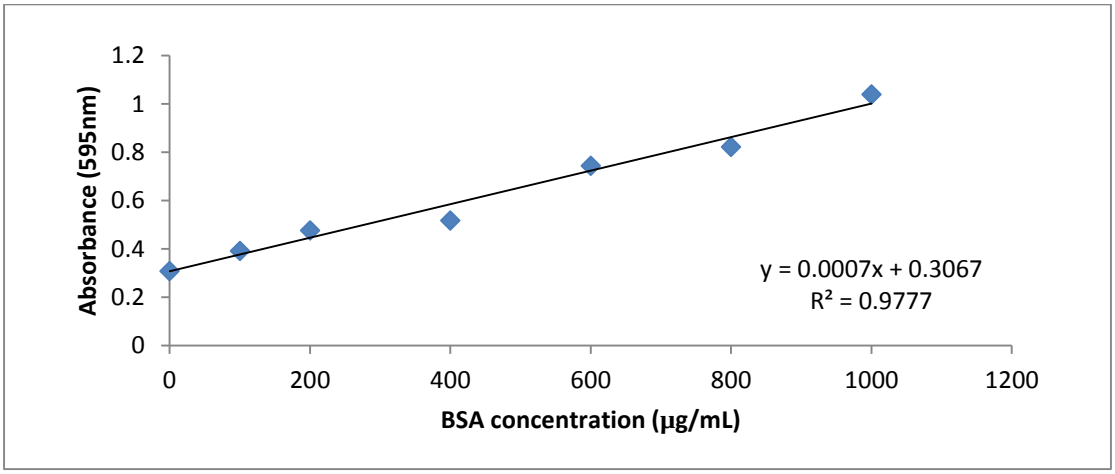
***50 mM ammonium acetate (pH 3.0 – 5.5)* – 100 mL**

- In 80mL dH_2O, 0.385 g of ammonium acetate was dissolved. The pH was adjusted to desired value using glacial acetic acid before making up to a final volume of 100 mL.

A4: Bradford's Protein Assay Standard Curve

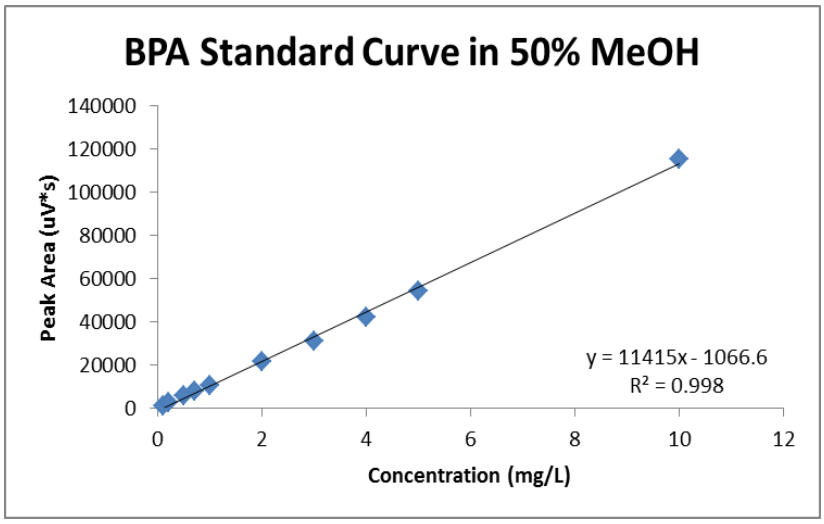
Below is an example of a Bradford's standard curve that was generated in this study.

Each time a Bradford's assay was performed, a new standard curve was generated.

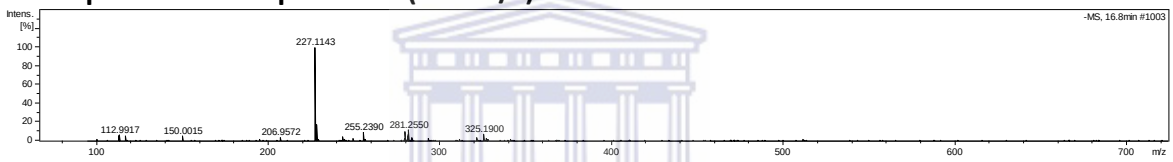


APPENDIX B

B.1 BPA standard curve – BPA standard prepared in 50% methanol

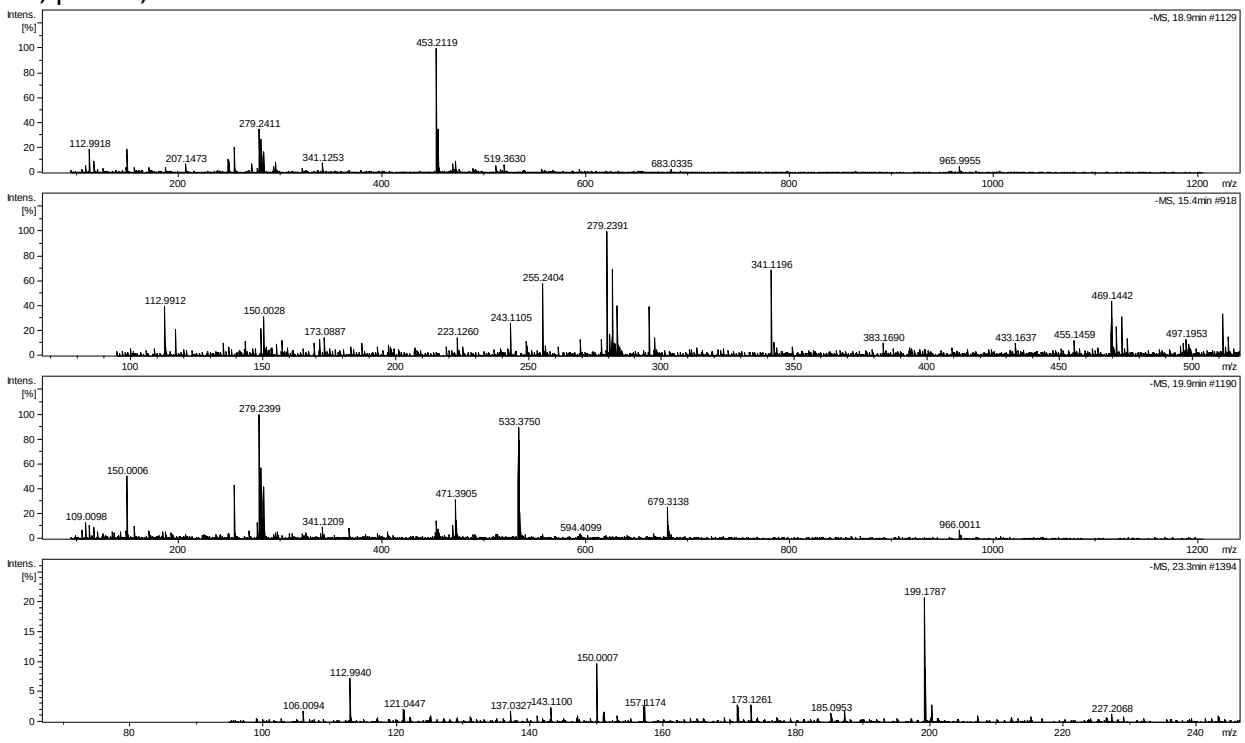


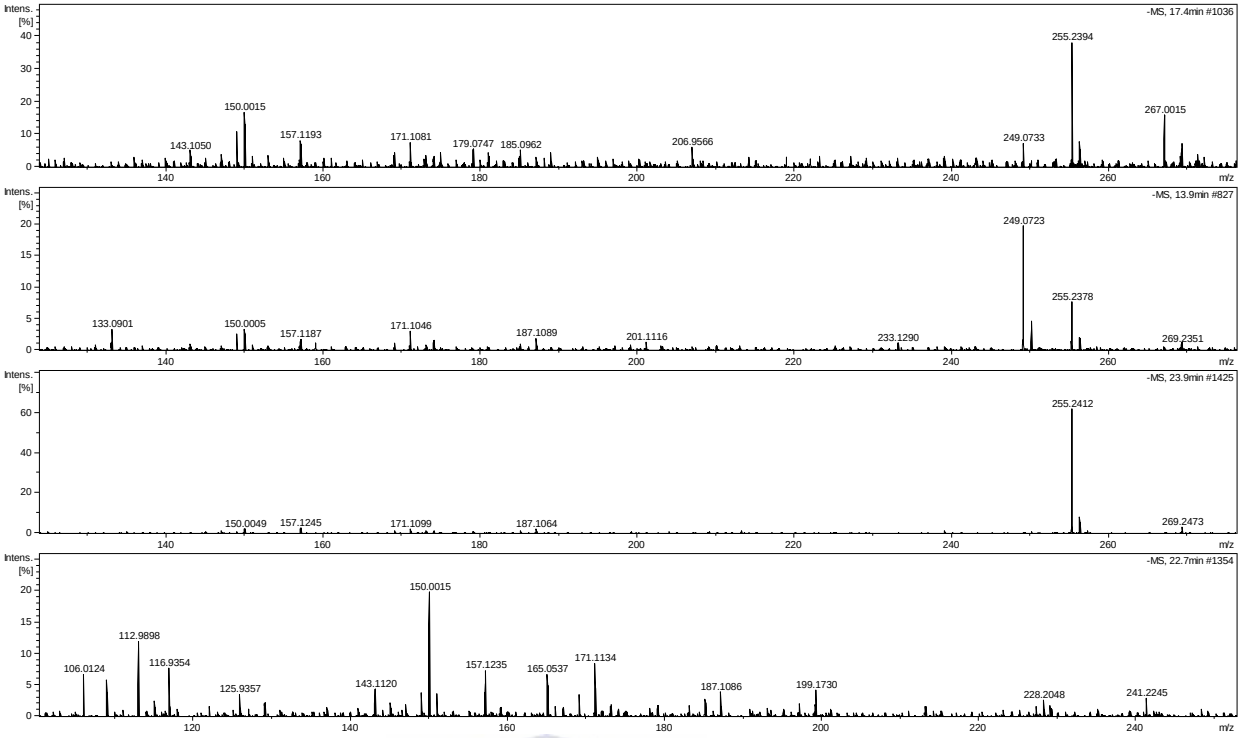
Mass spectrum of bisphenol-A (227 m/z)



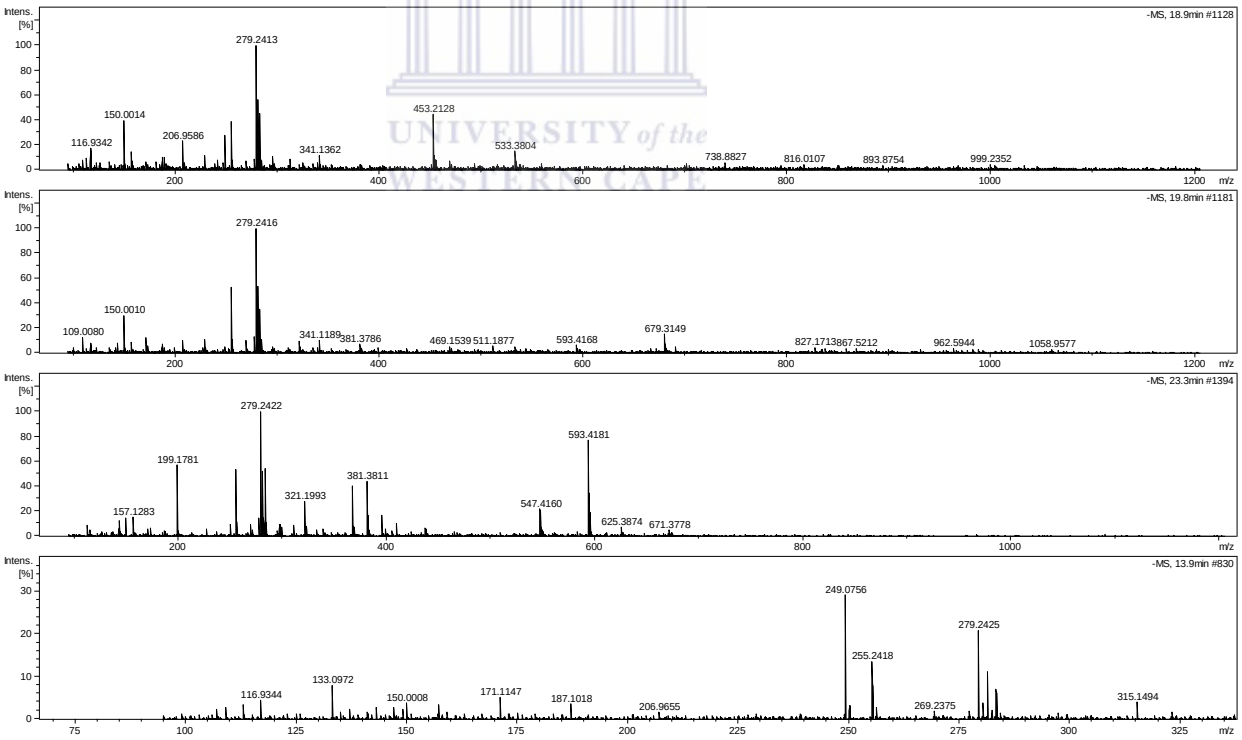
B.2 LC-MS profiles of the putative BPA metabolites - Free laccase

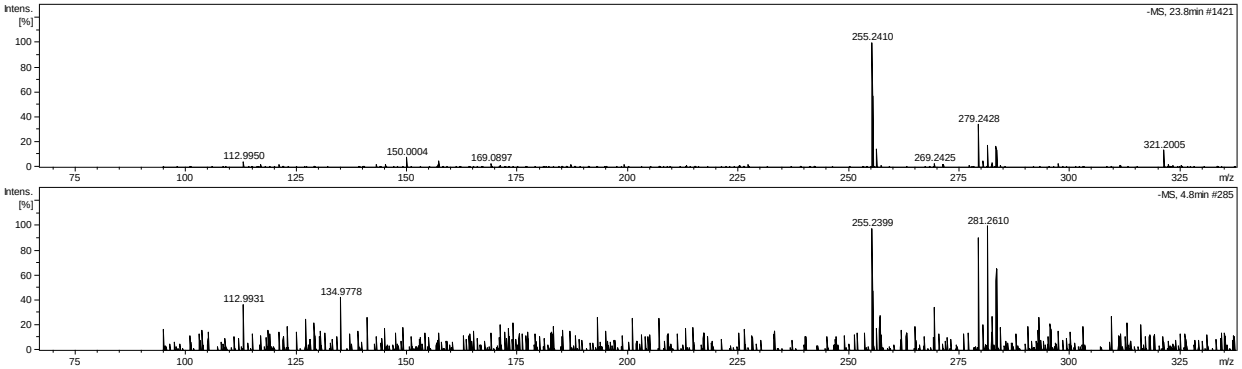
TvL; pH 5.5; 30˚C



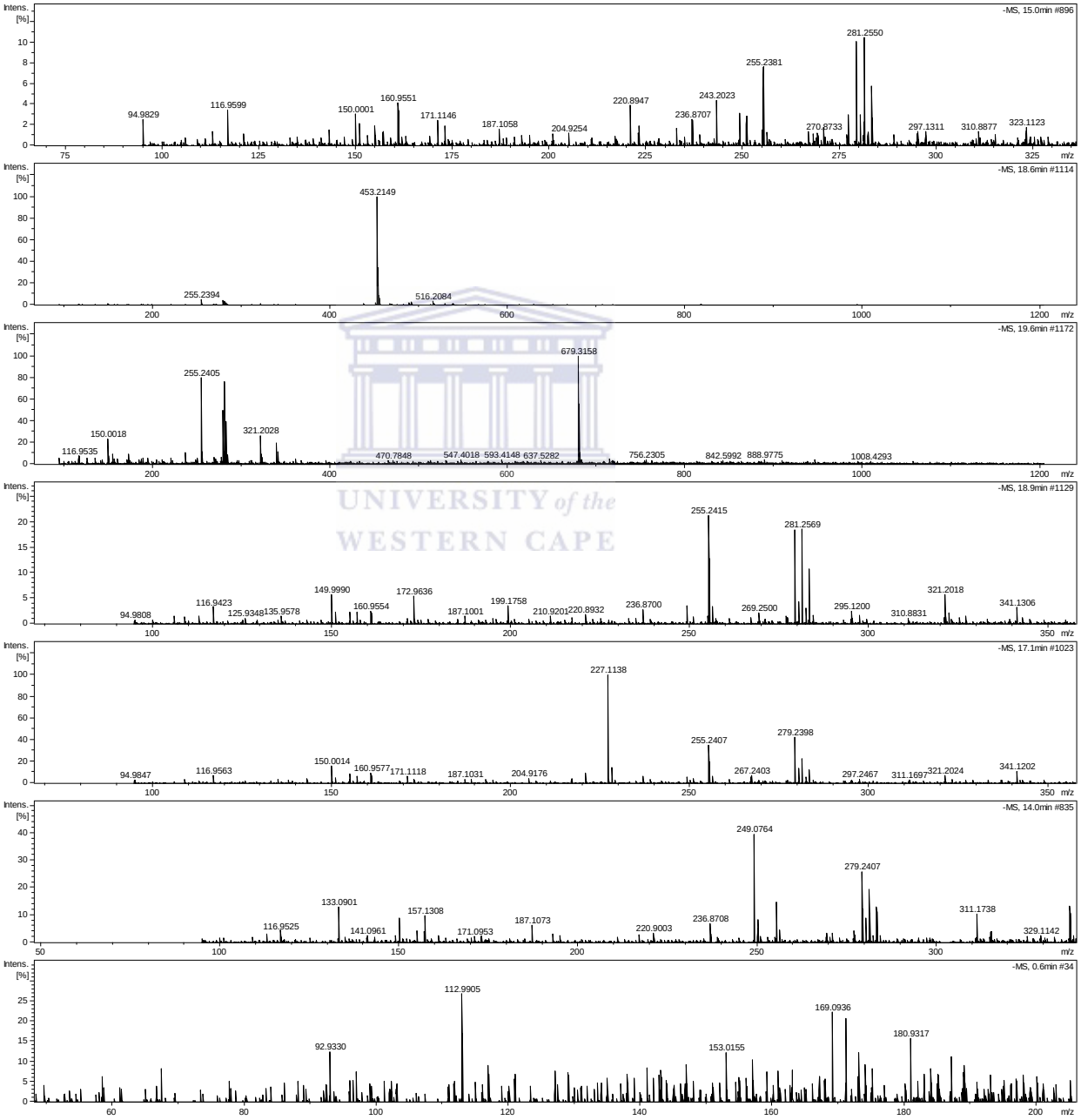


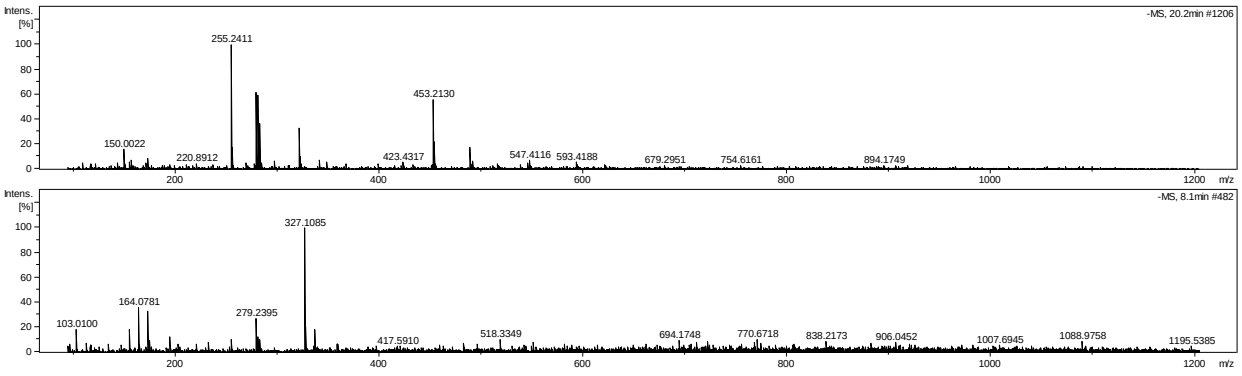
TpL, pH 4.5; 30˚C



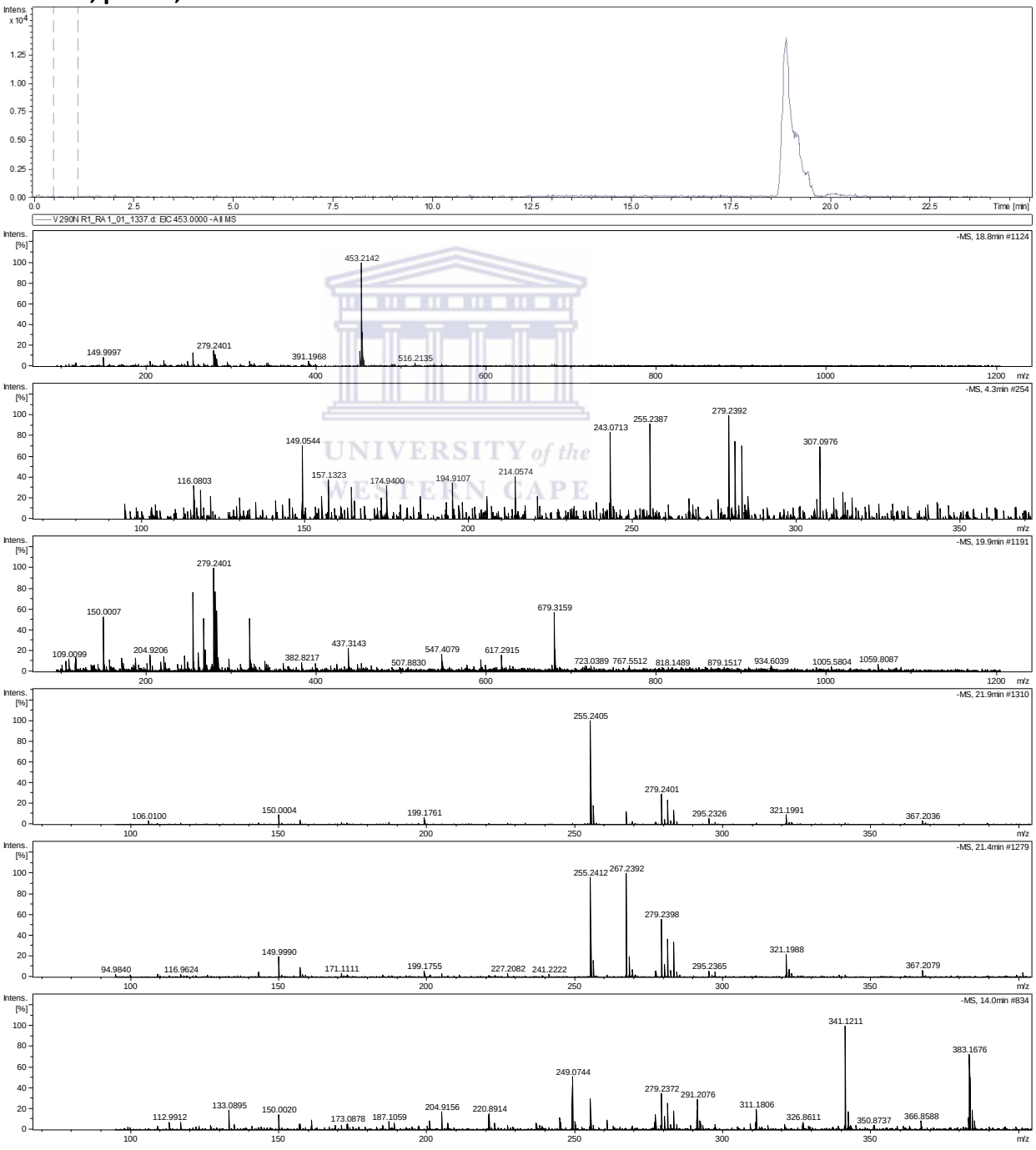


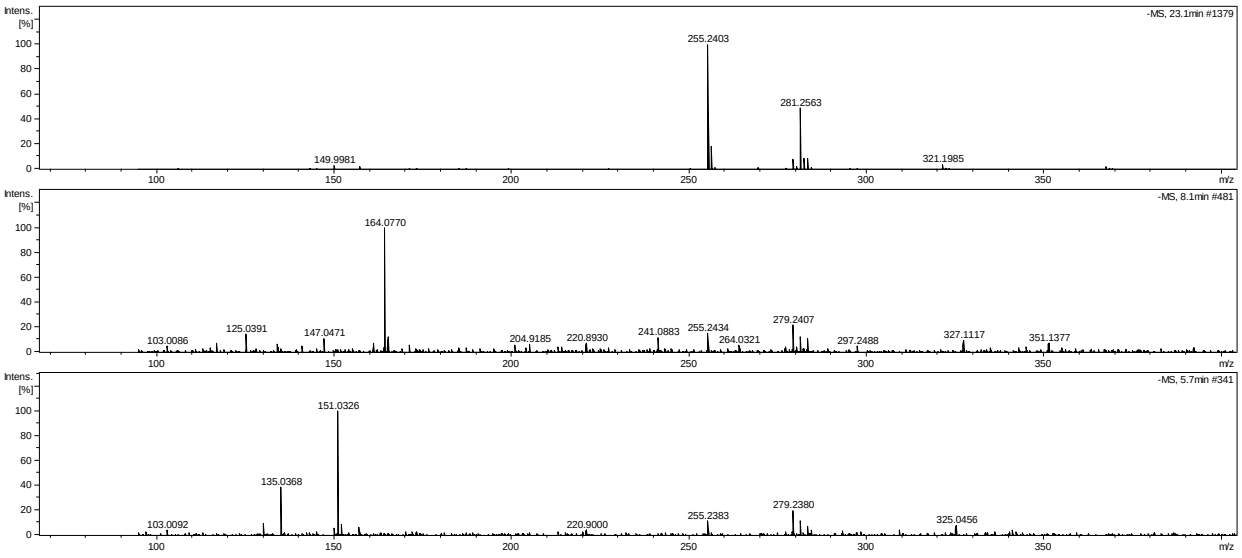
SLAC, pH 9.5; 30˚C





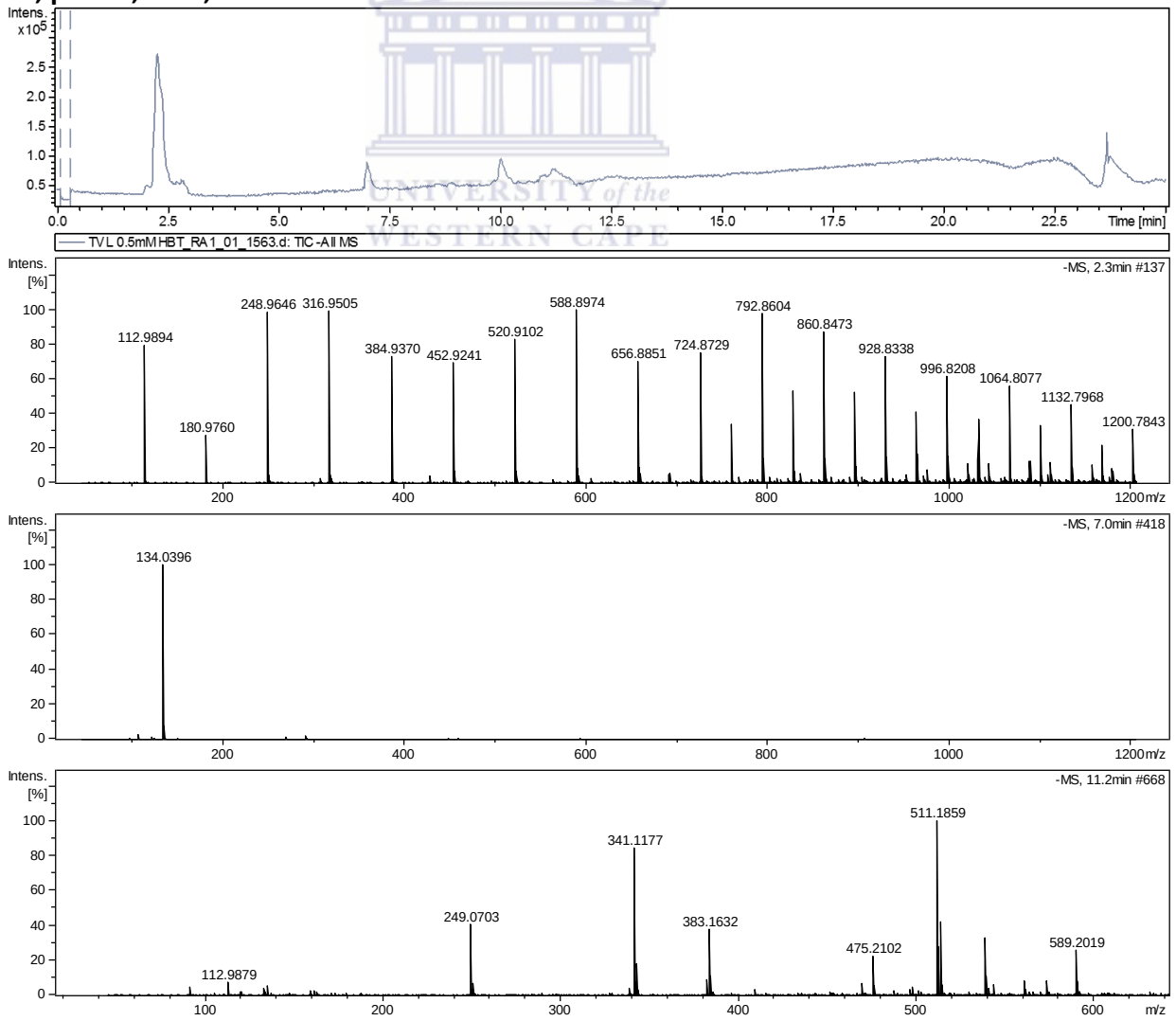
SLAC-VN, pH 7.0; 40˚C

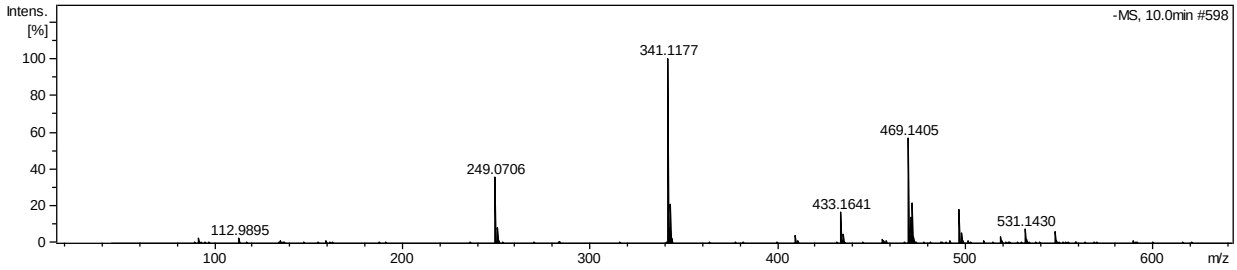




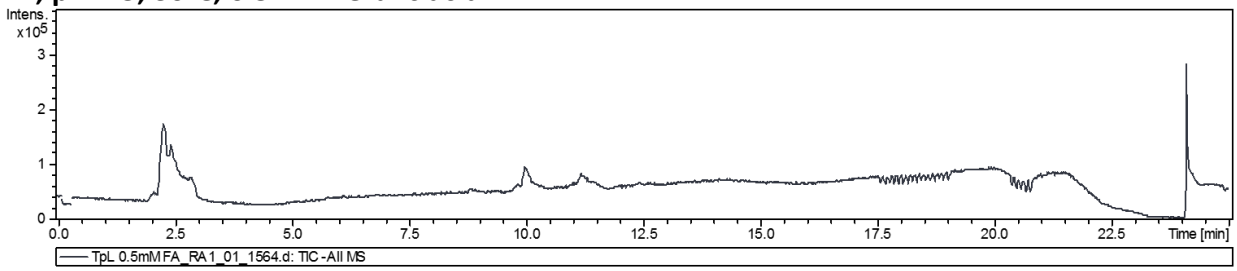
B.3 LC-MS profiles of the putative BPA metabolites – Free laccase in the presence of redox mediators

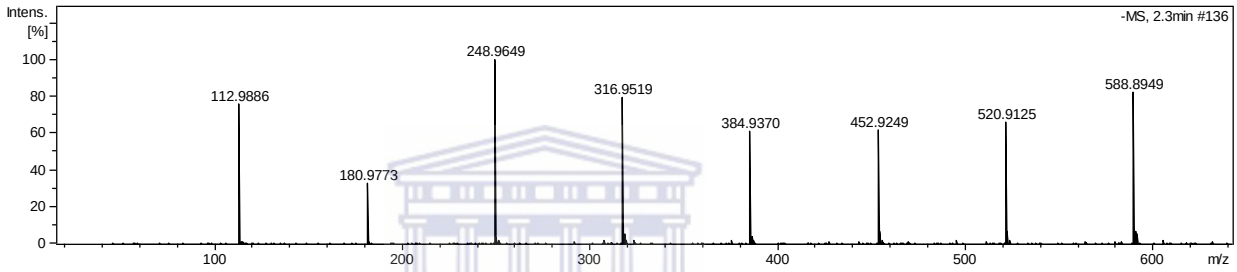
TvL, pH 5.5; 30˚C; 0.5 mM HBT

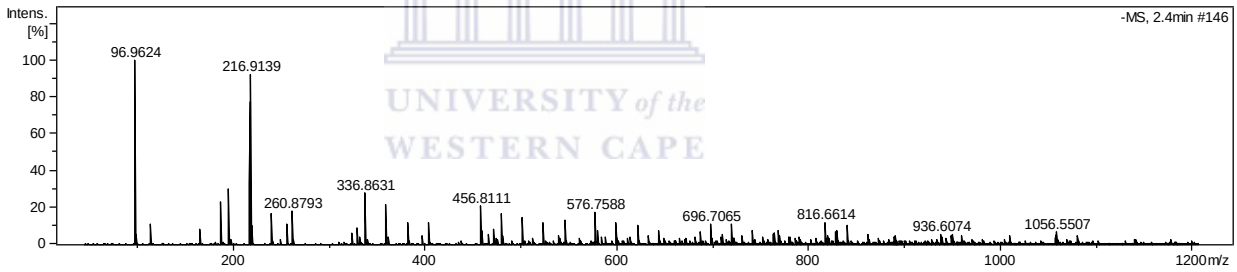


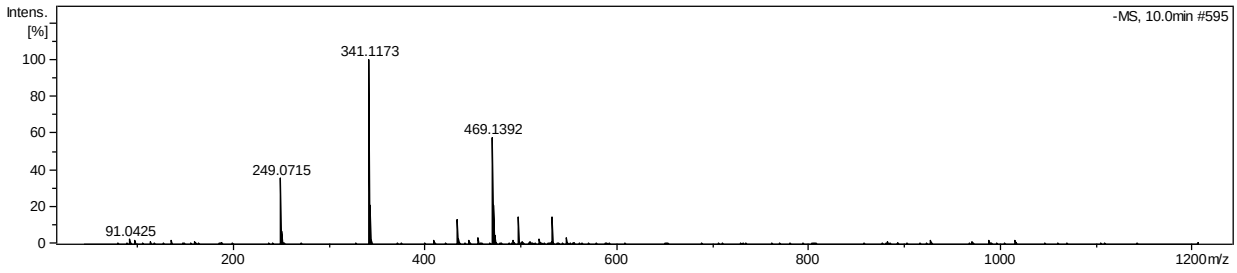


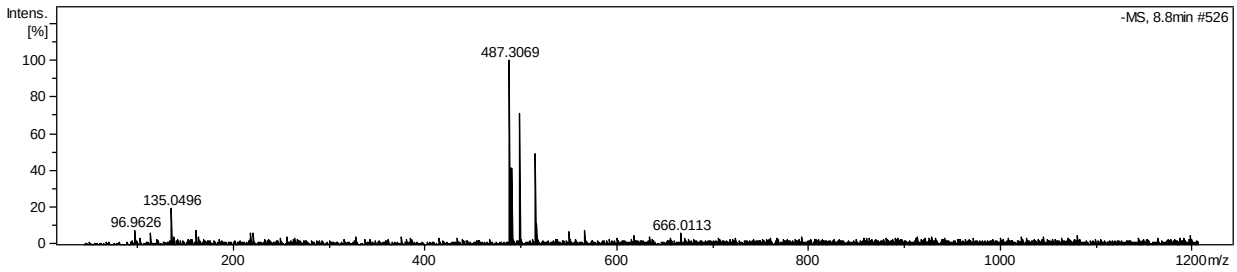
TvL; pH 4.5; 30˚C; 0.5mM Ferulic acid

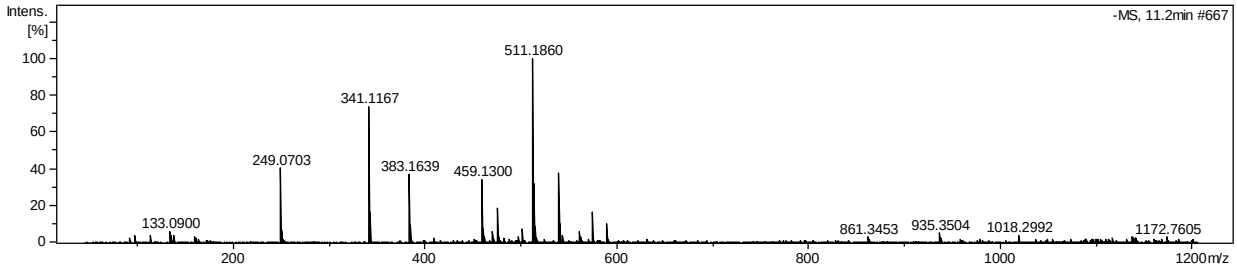




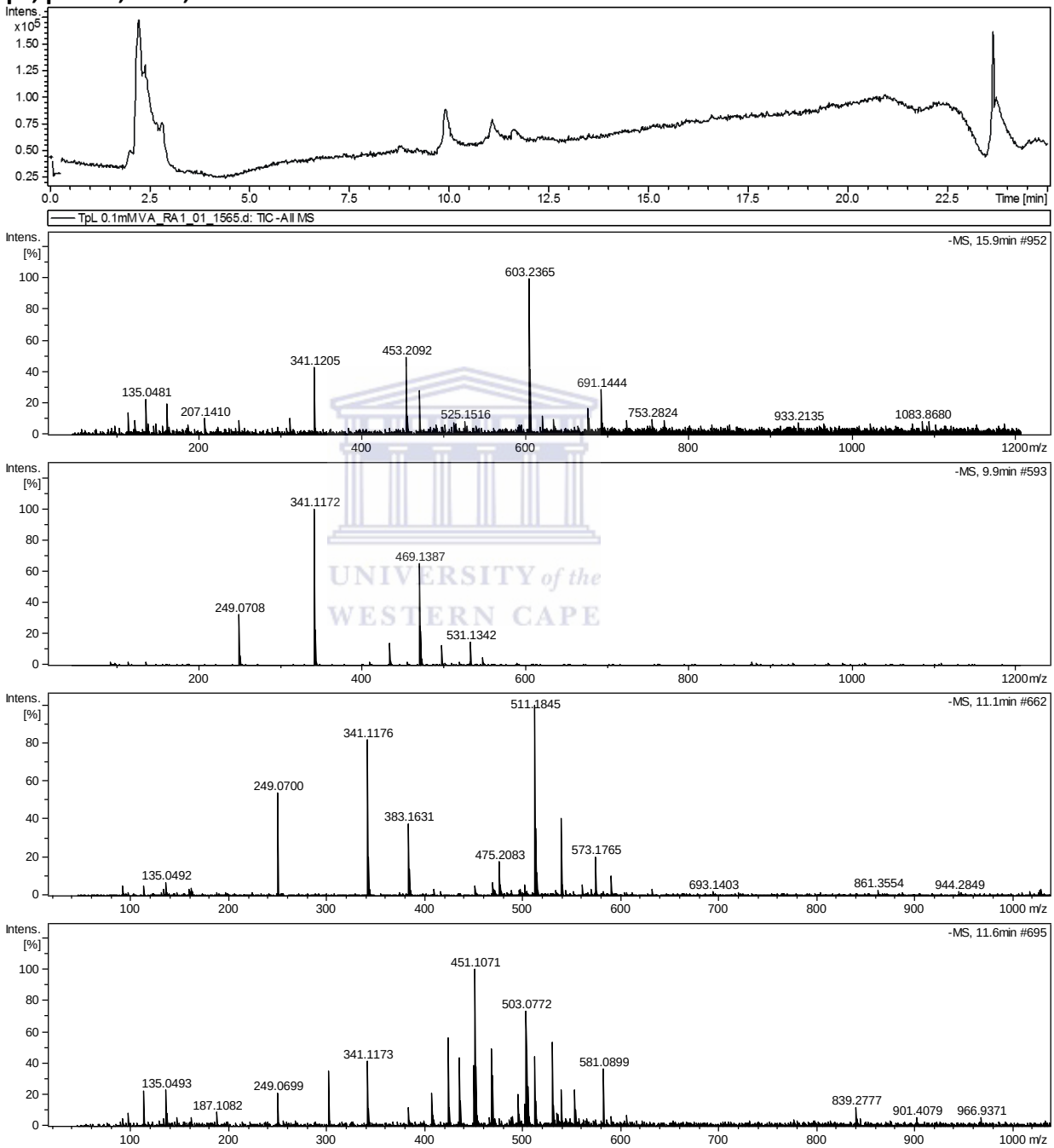




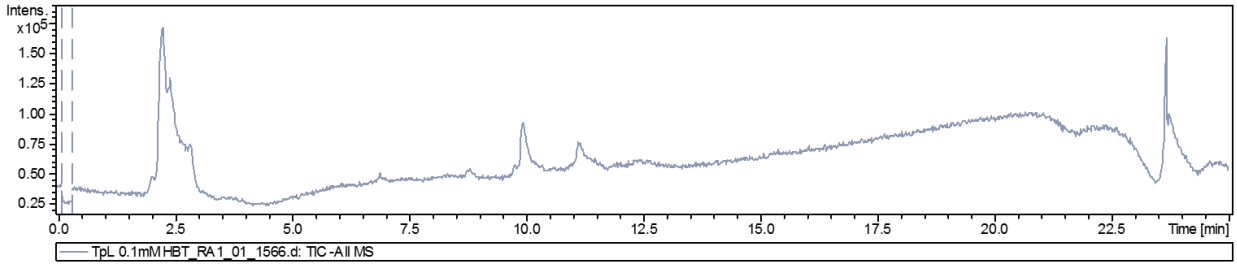


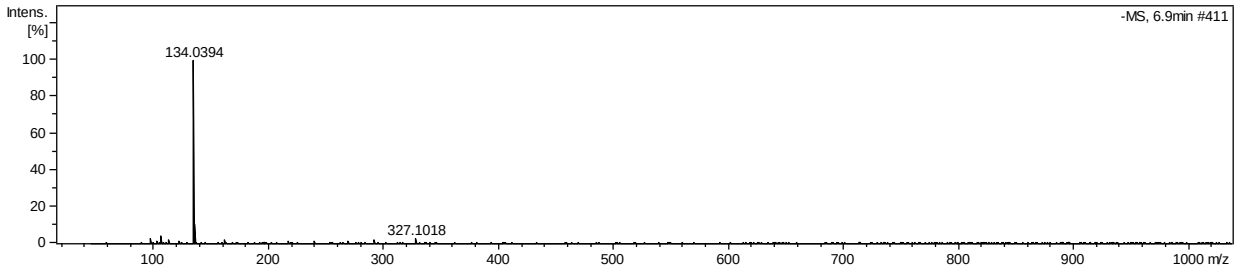


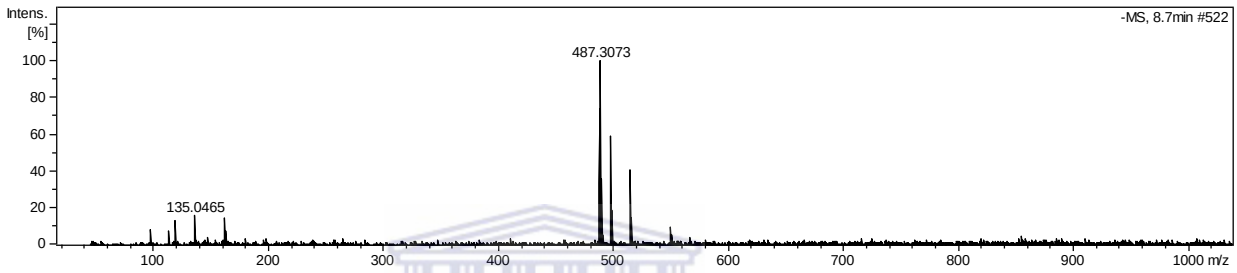
TpL, pH 4.5; 30˚C; 0.1mM VA

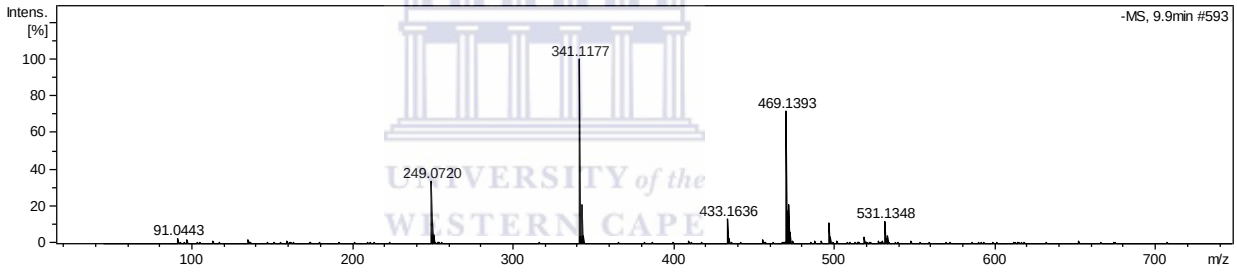


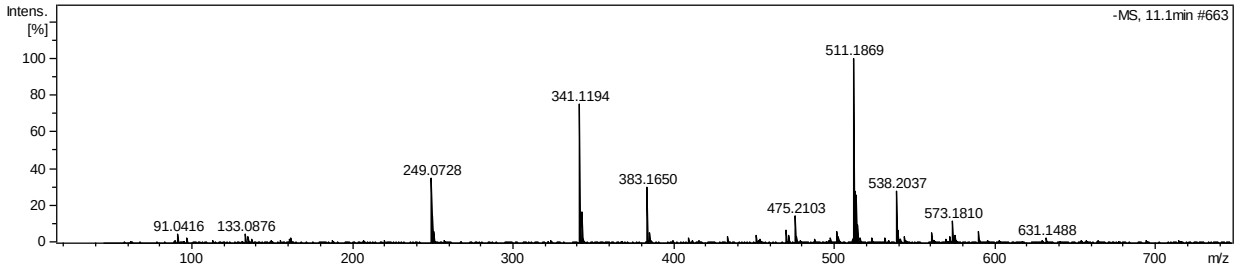
TpL, pH 4.5, 30˚C; 0.1mM HBT



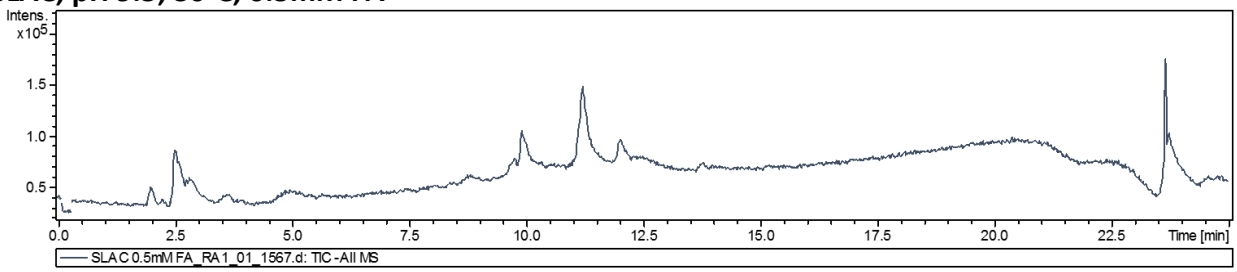


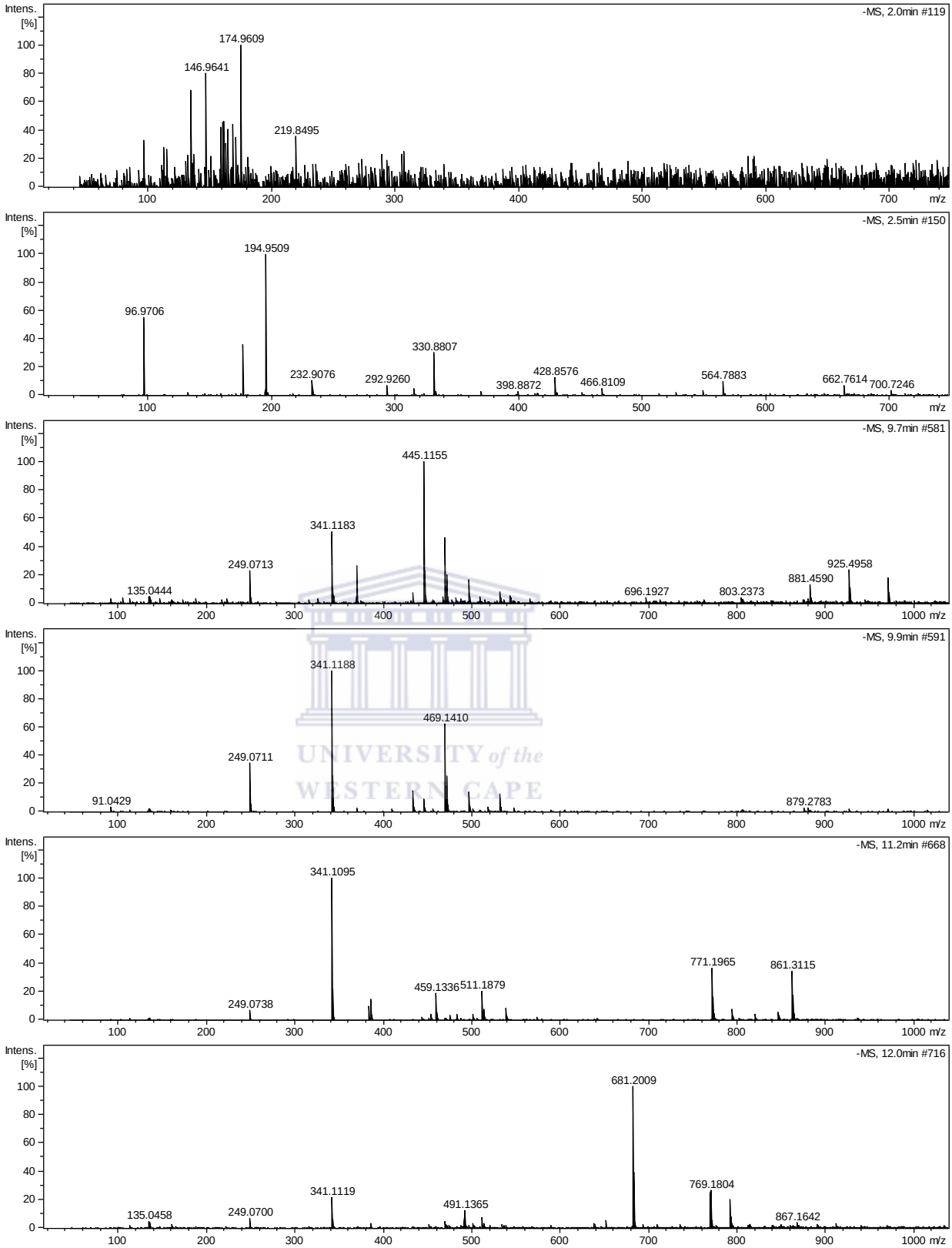


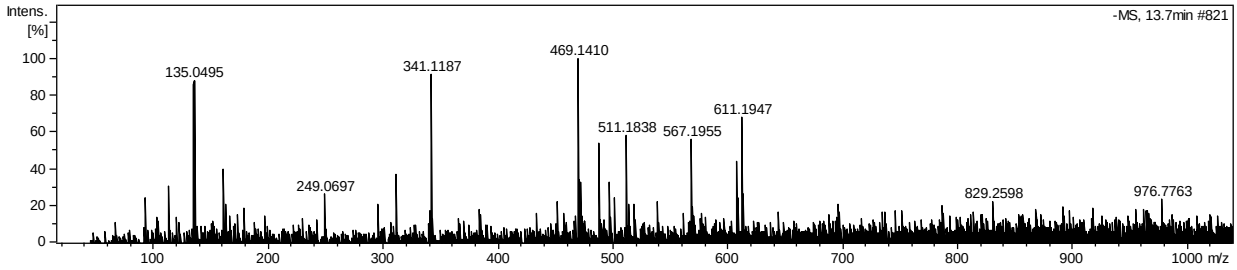




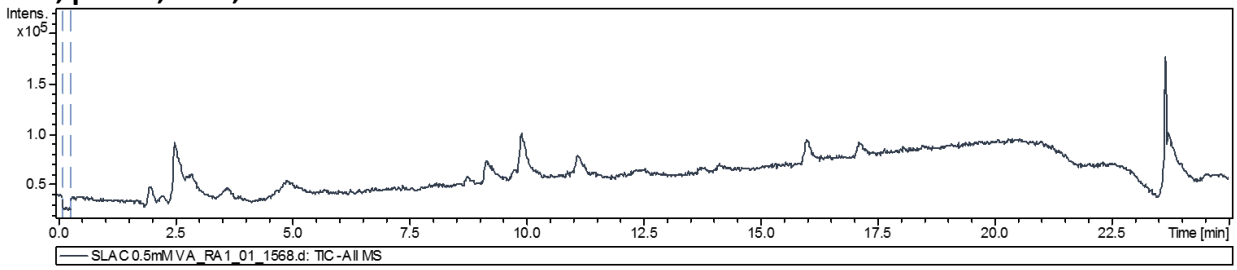
SLAC, pH 9.5; 30˚C, 0.5mM FA

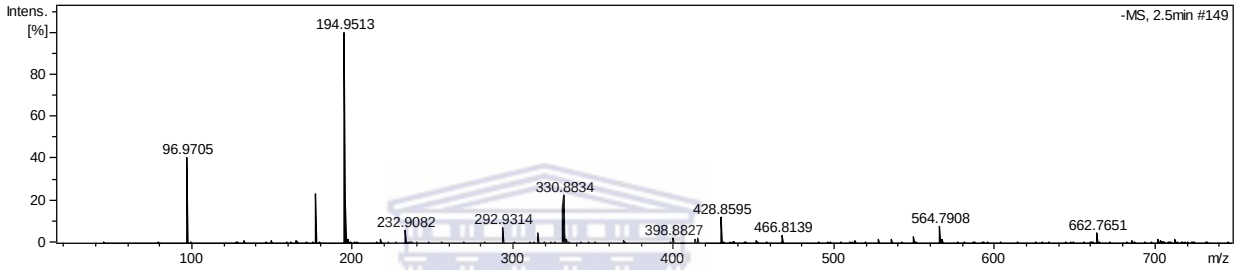


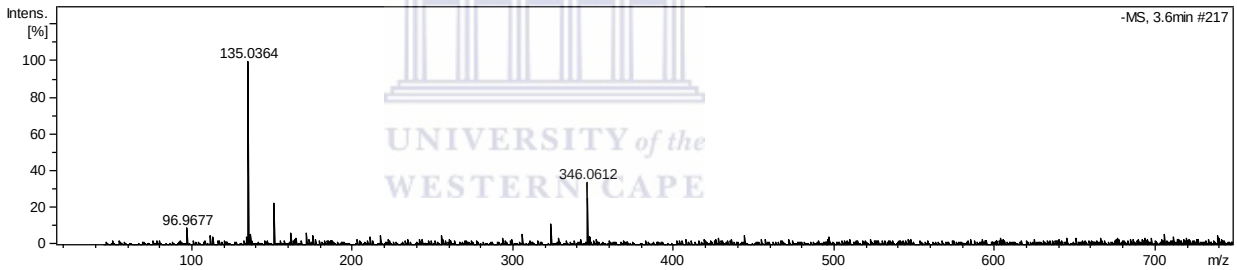


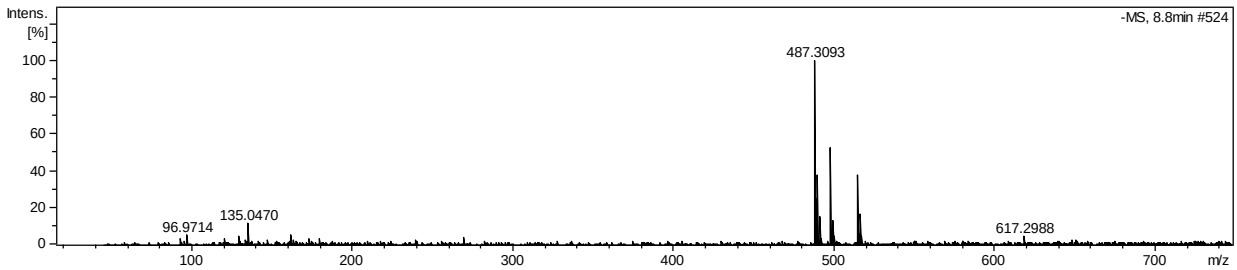


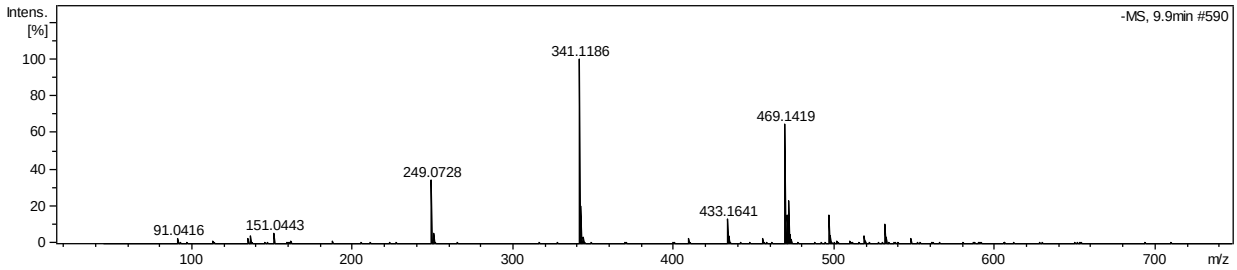
SLAC, pH 9.5, 30˚C, 0.5mM VA

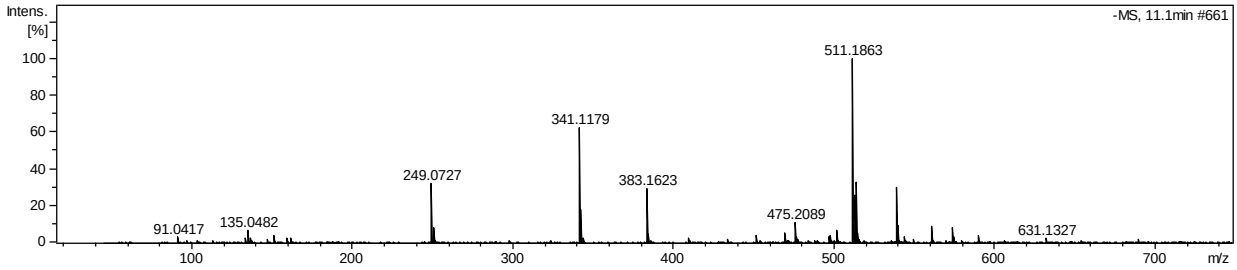


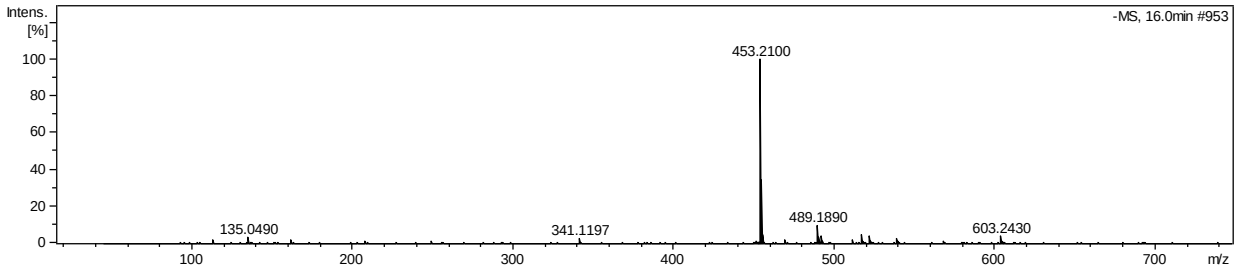


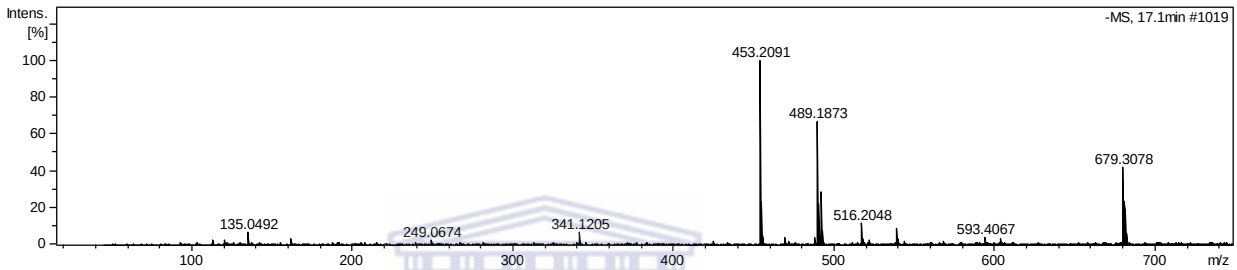




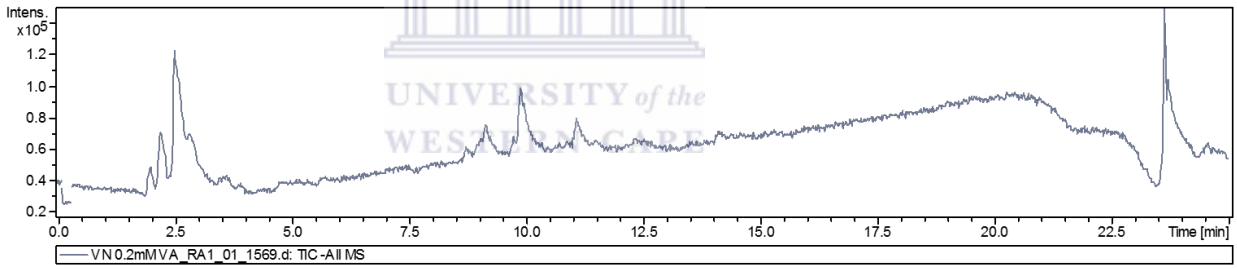


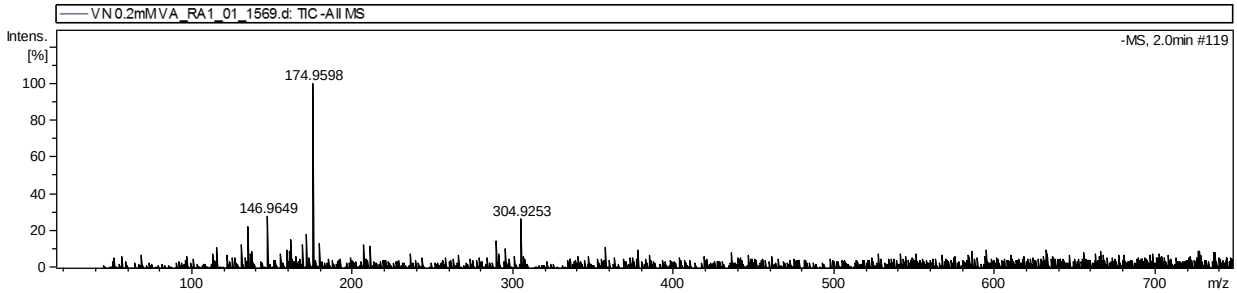


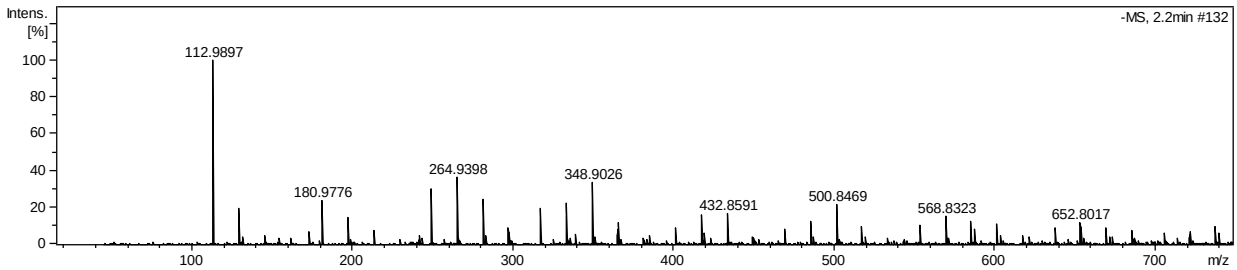


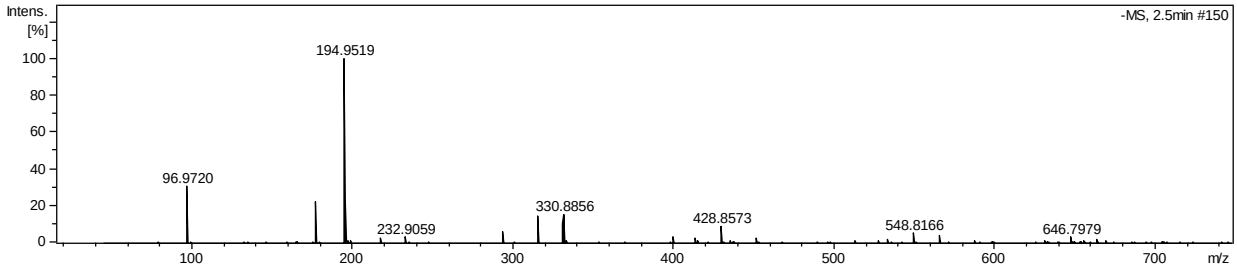


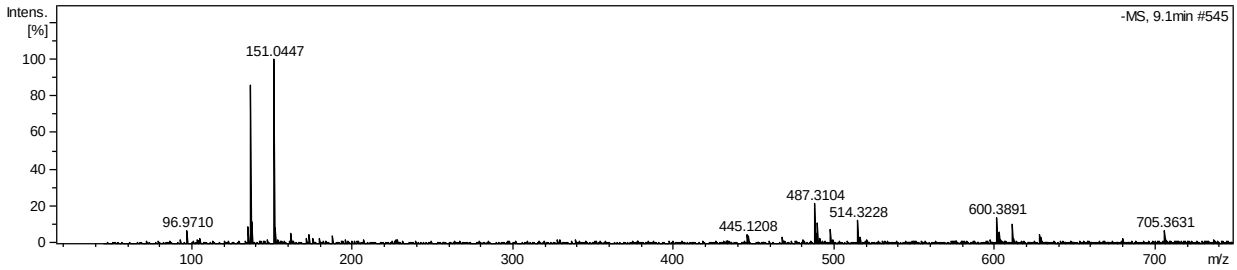
SLAC-VN, pH 7.0, 40˚C, 0.5MM VA

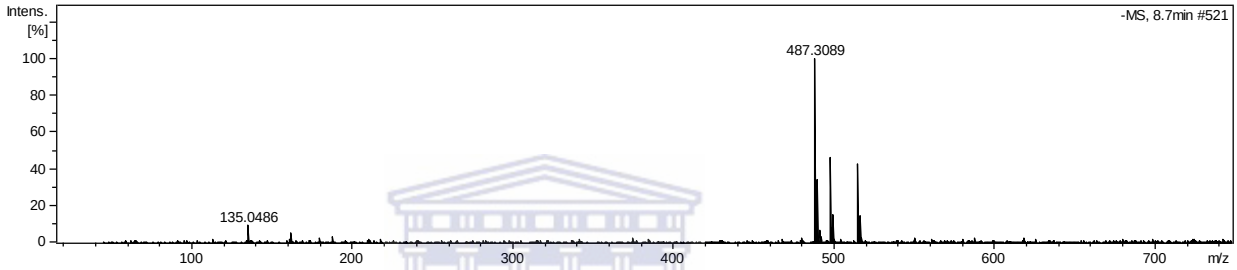


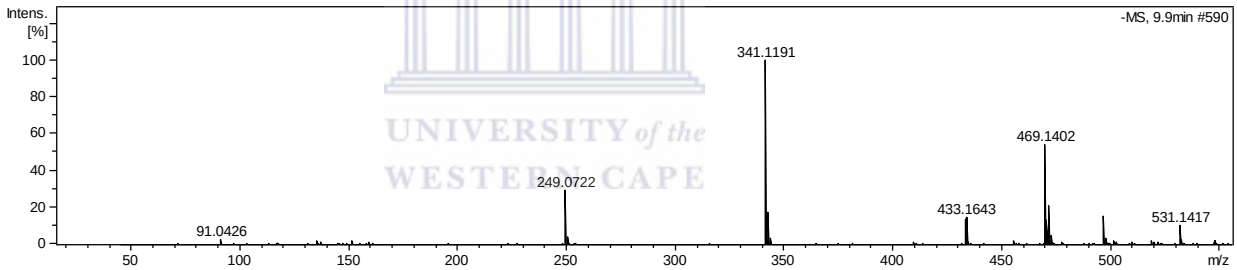


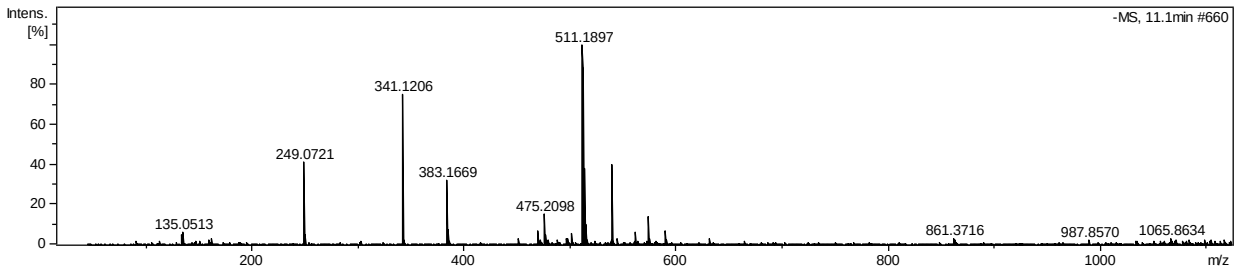






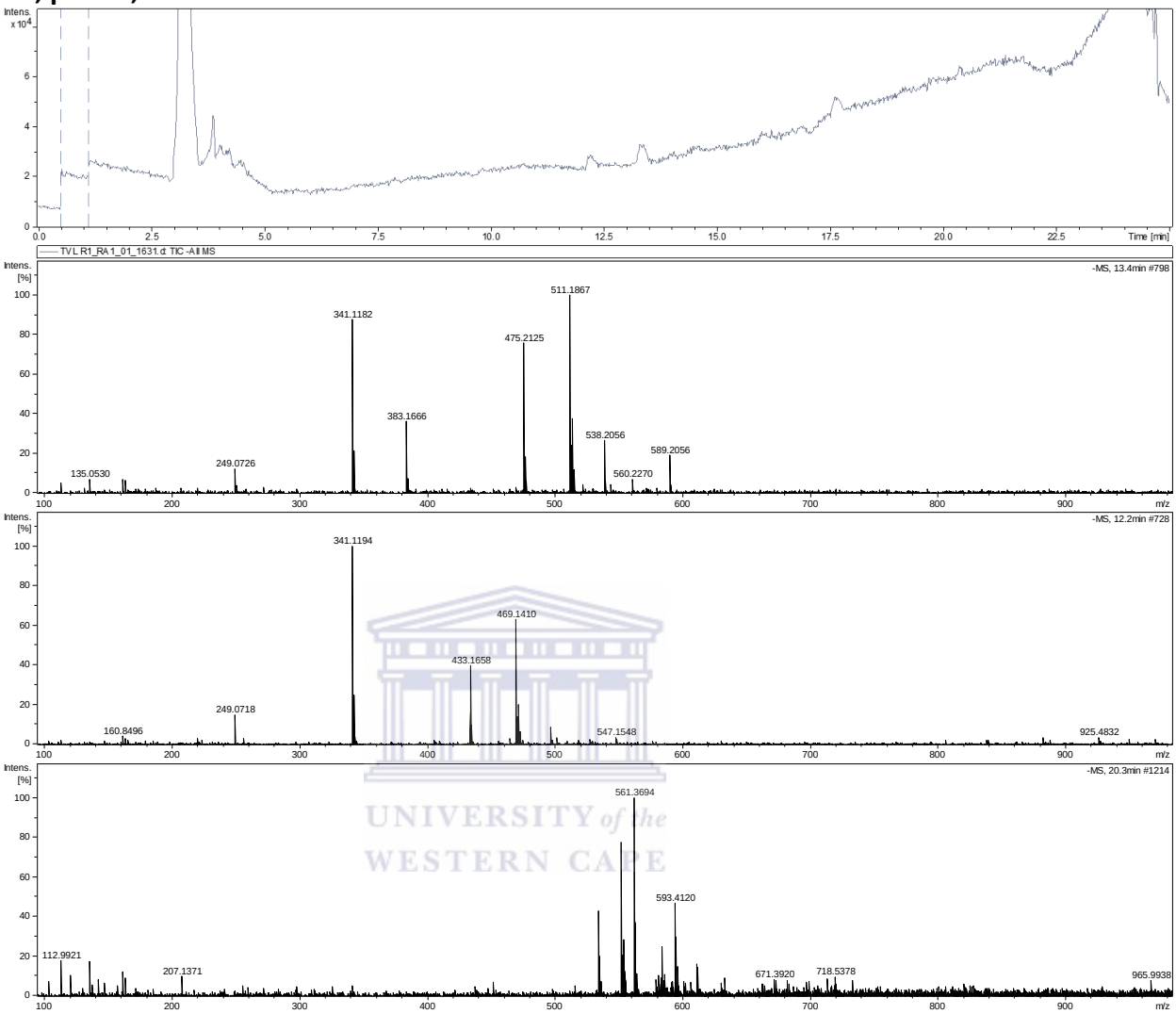




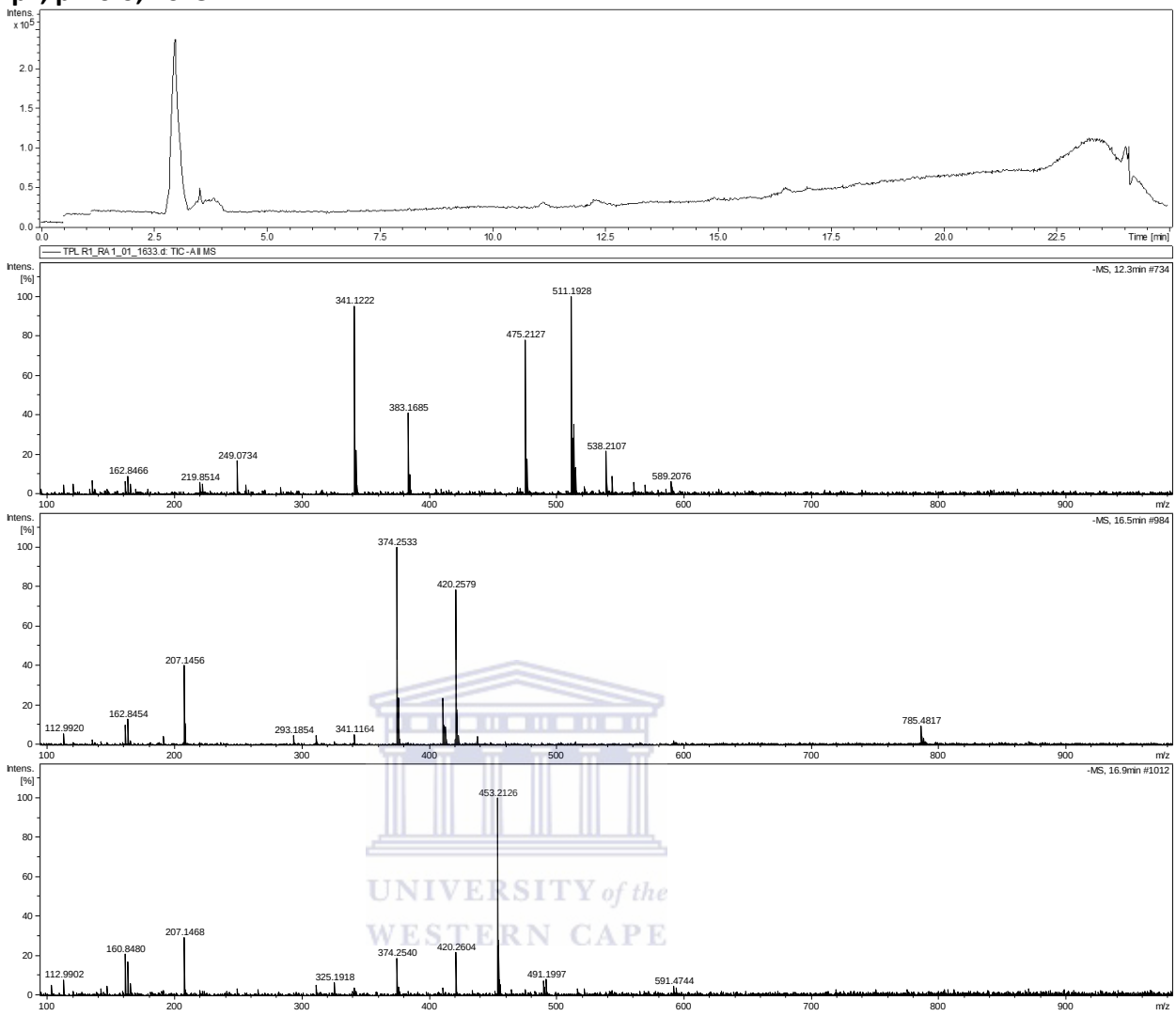


B4. LC-MS profiles of the putative BPA metabolites – Encapsulated laccase in the absence of a redox mediator

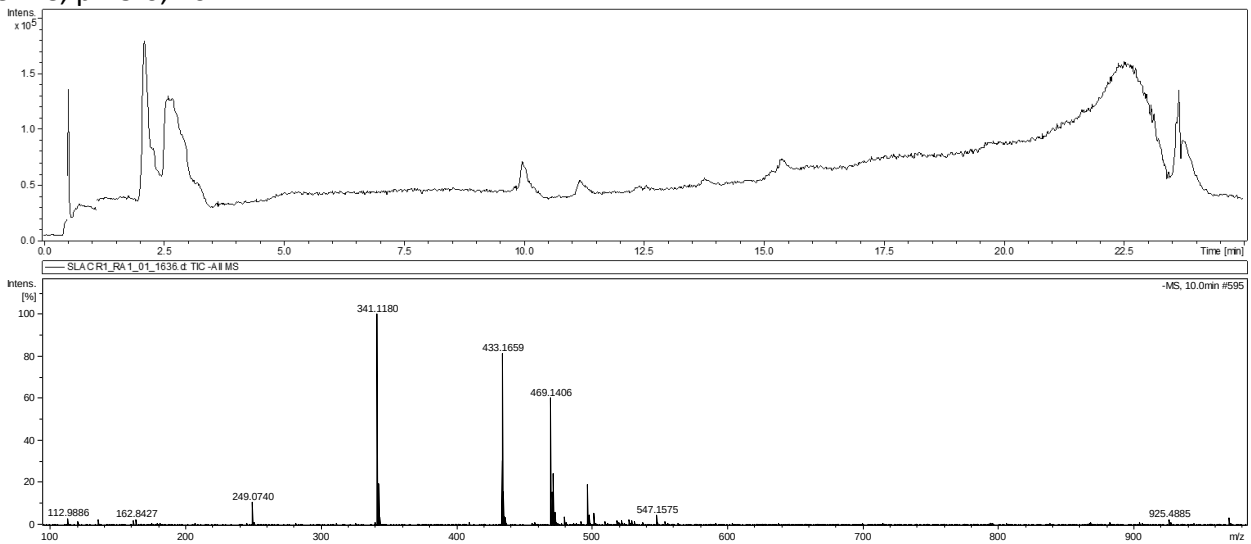
TvL, pH 5.0; 40°C

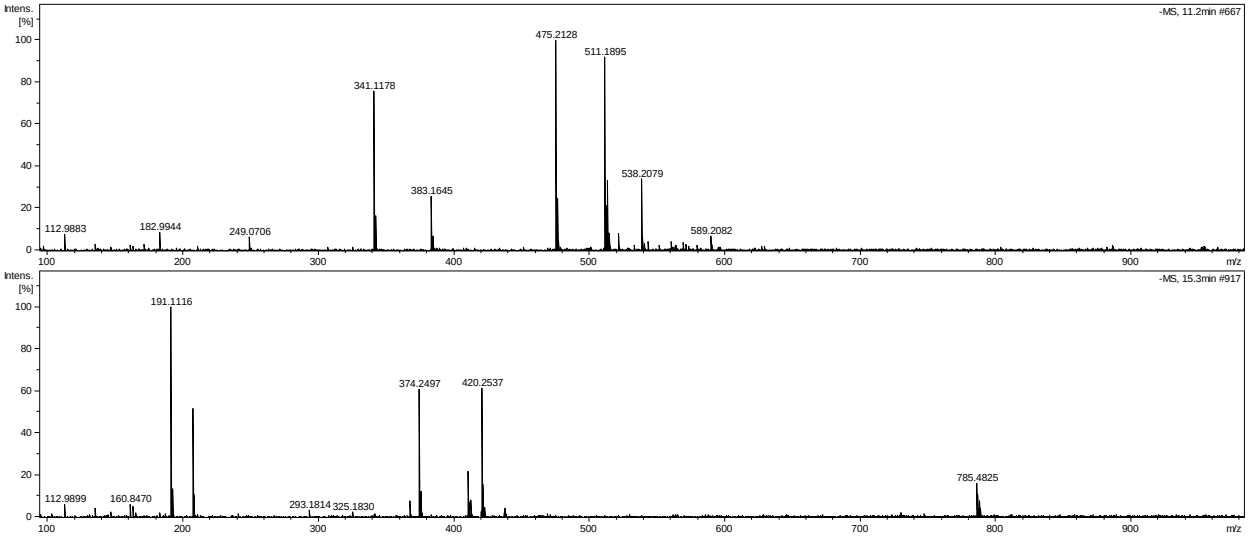


TpL; pH 6.0; 40°C

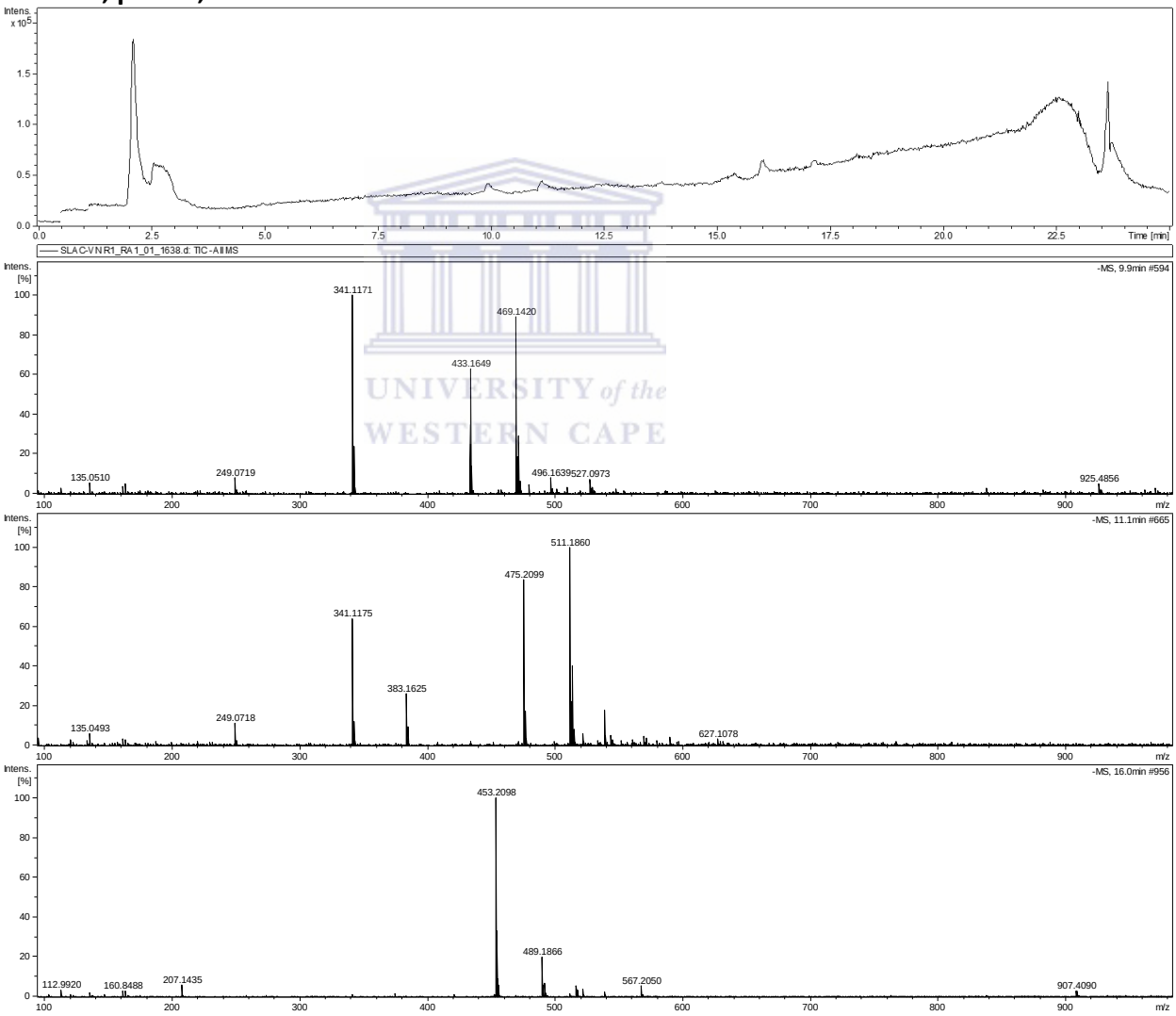


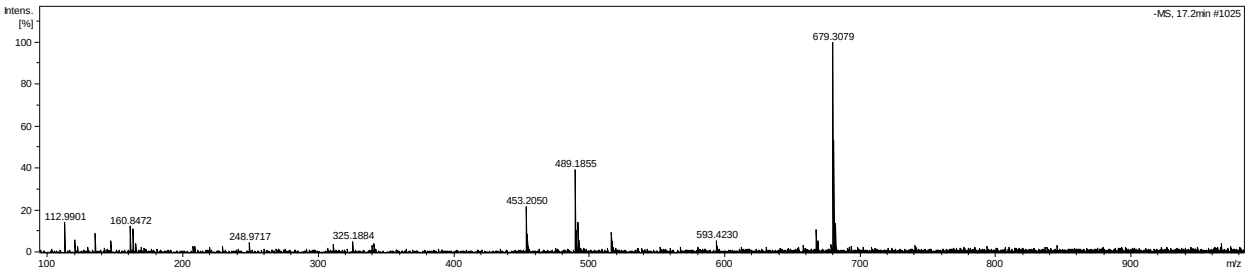
SLAC; pH 9.0; 40





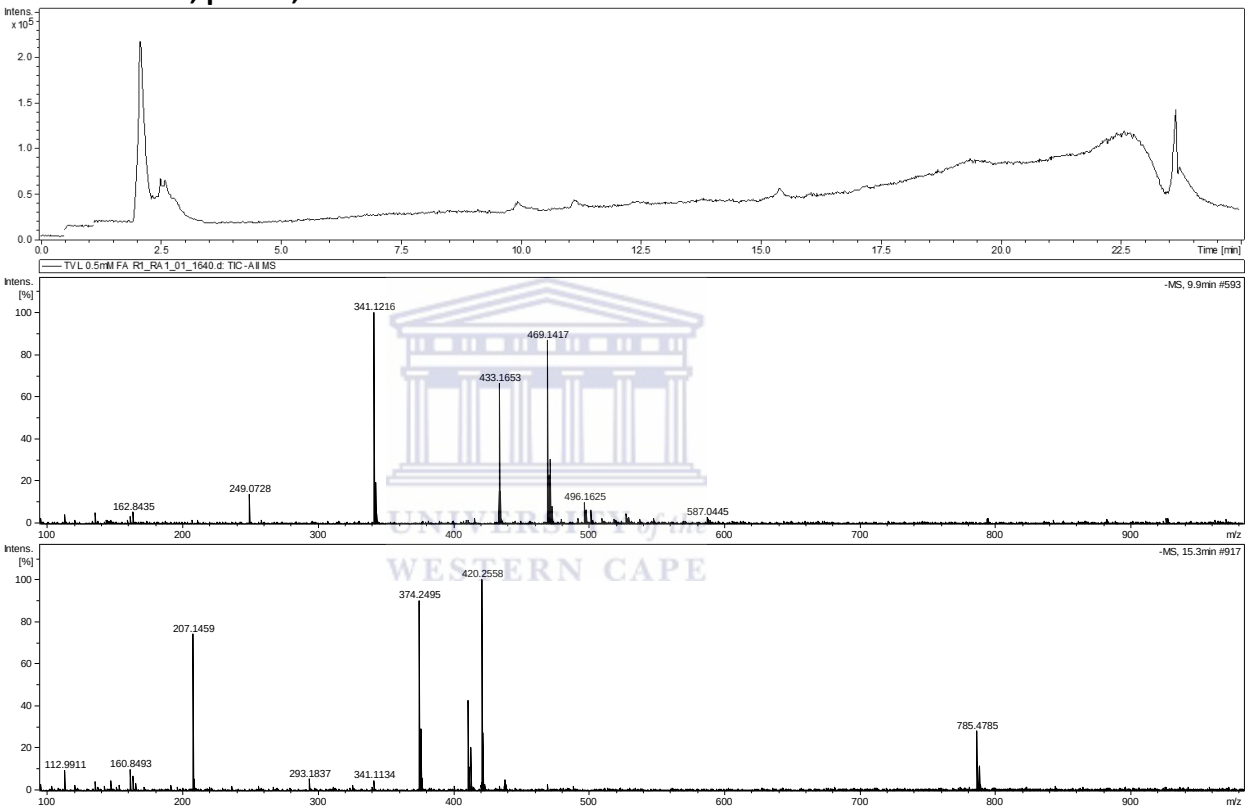
SLAC-VN; pH 7.0; 40°C



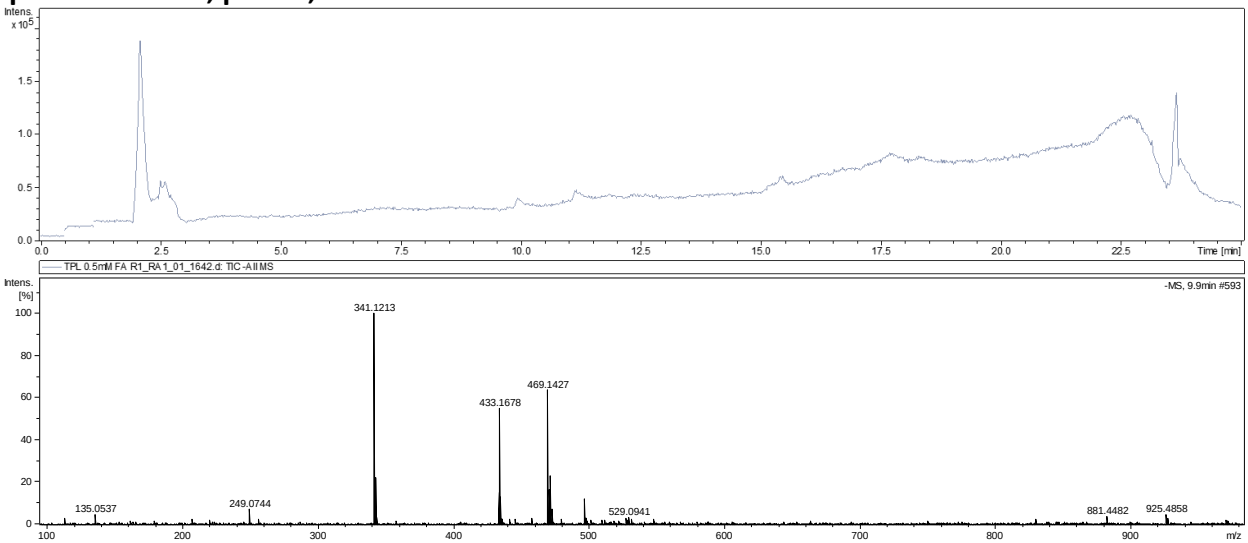


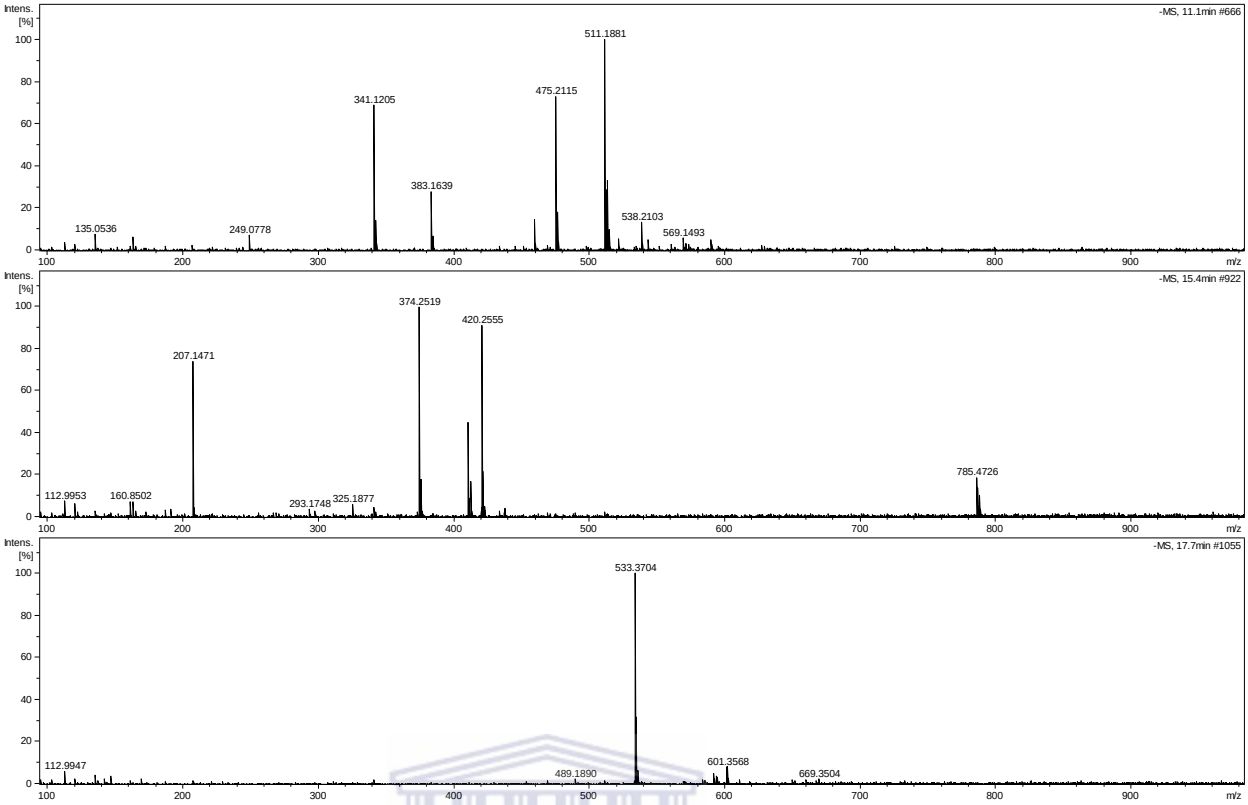
B5. LC-MS profiles of the putative BPA metabolites – Encapsulated laccase in the presence of redox mediators

TvL+0.5mM FA; pH 5.0; 40°C

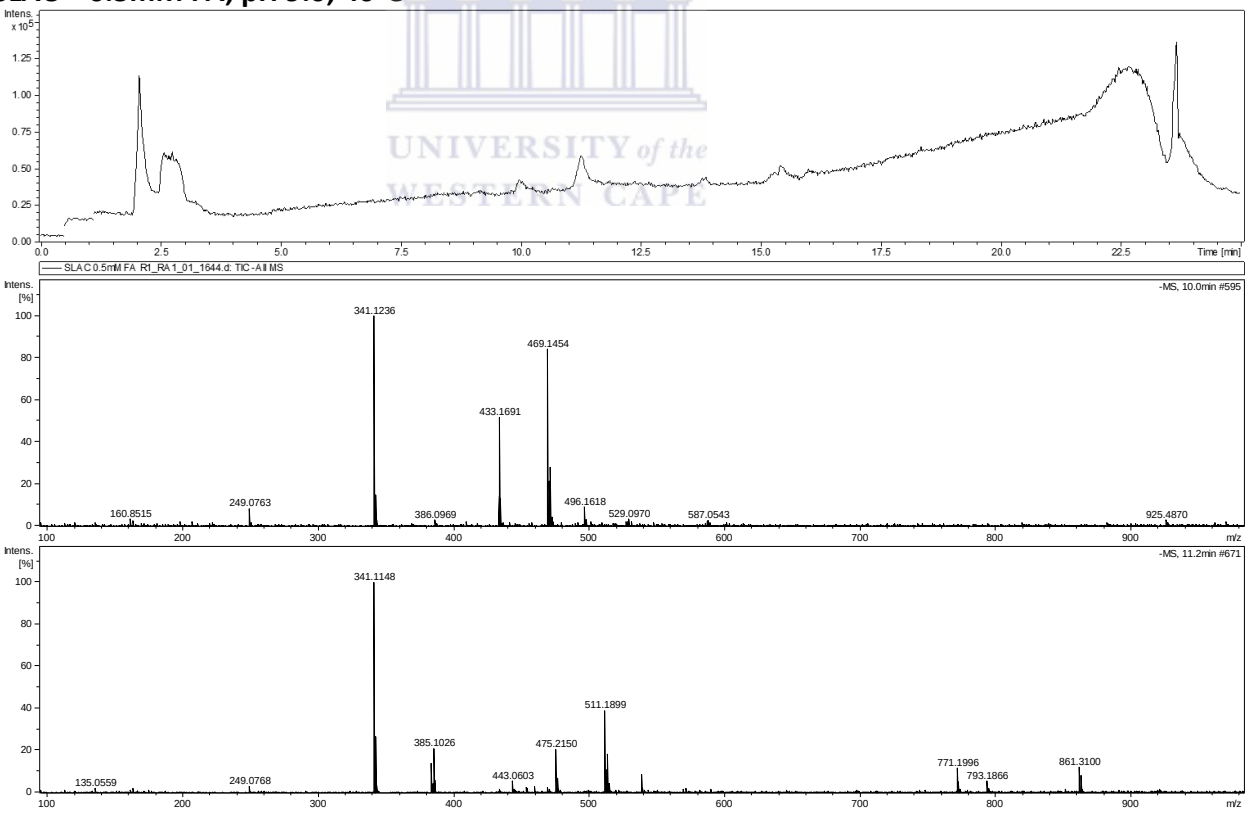


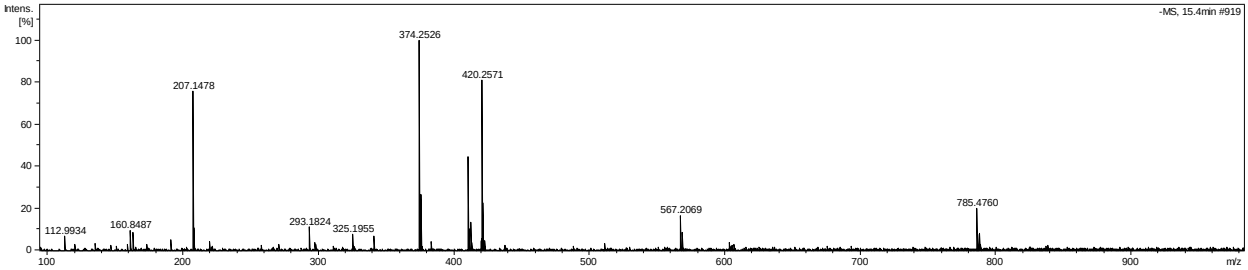
TpL + 0.5mM FA; pH 6.0; 40°C



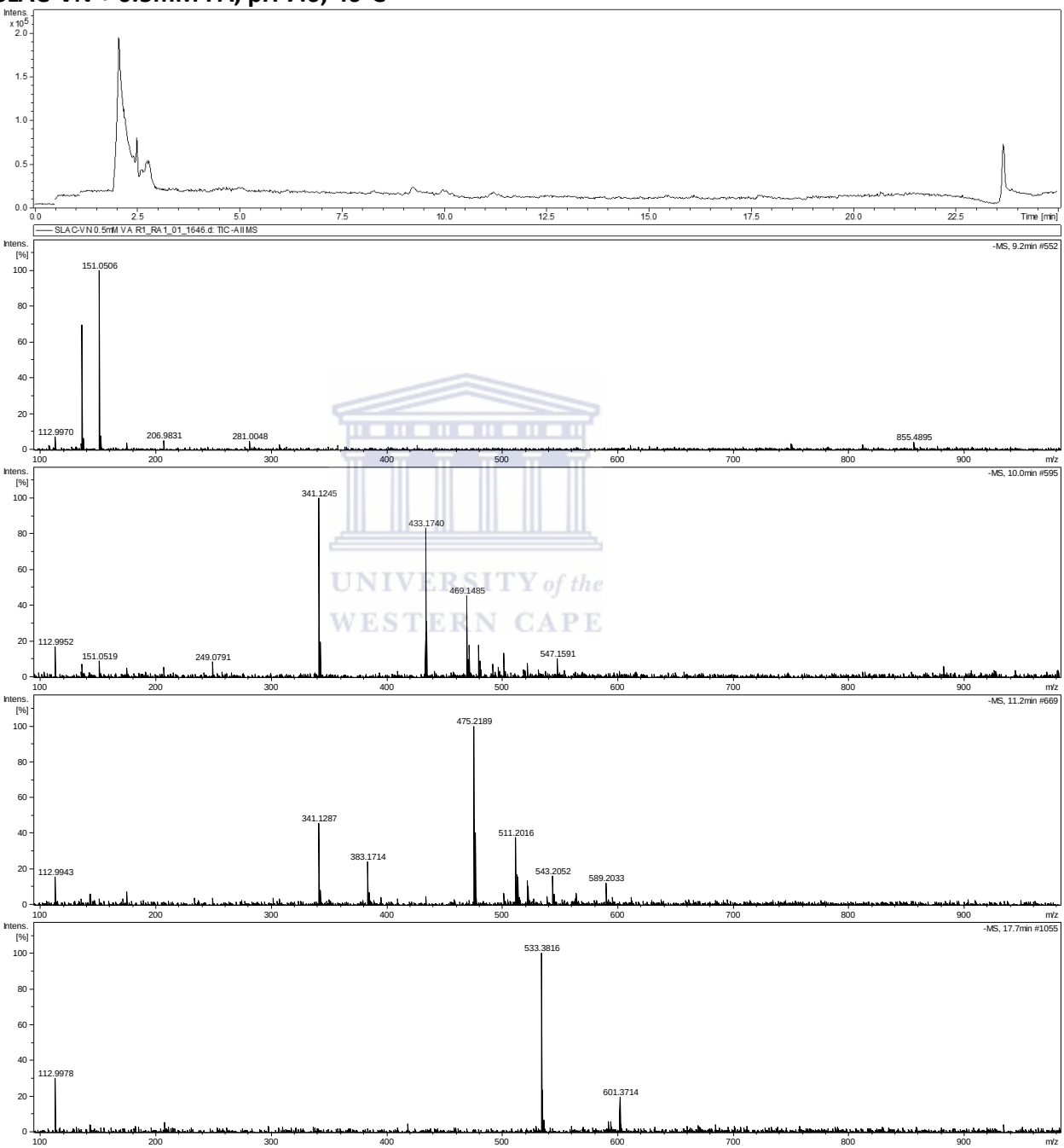


SLAC + 0.5mM FA; pH 9.0; 40°C



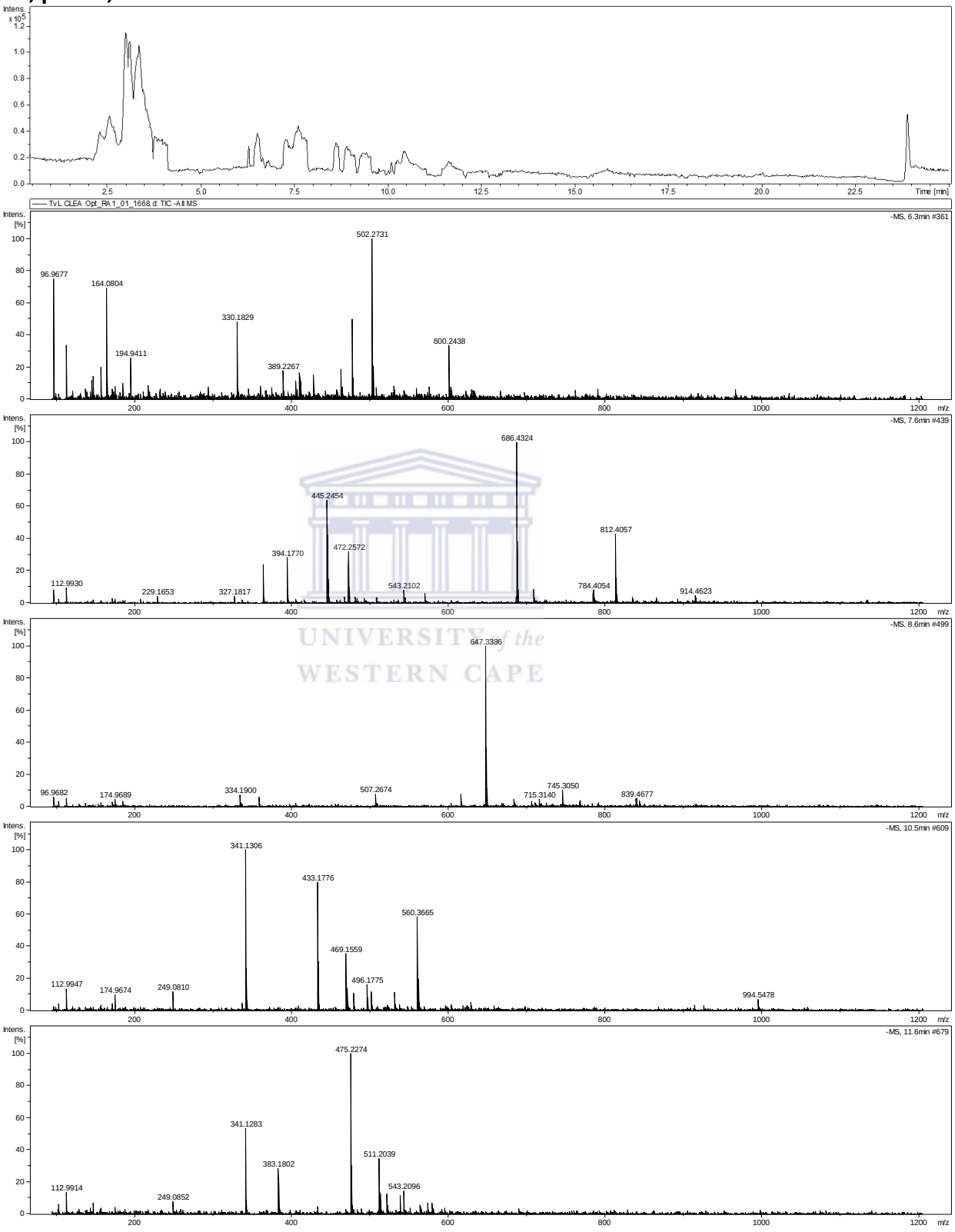


SLAC-VN + 0.5mM FA, pH 7.0; 40°C

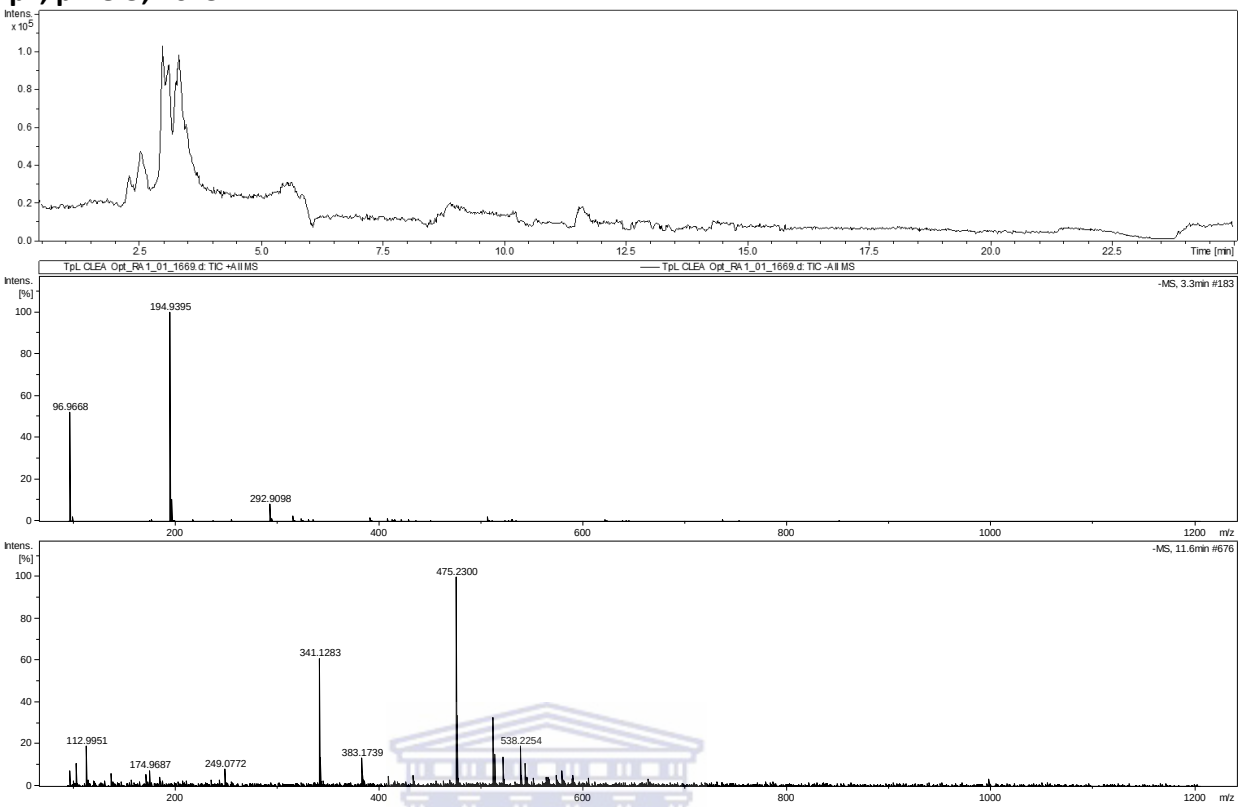


B6: LC-MS profiles of putative BPA metabolites – Laccase CLEAs in the absence of redox mediators

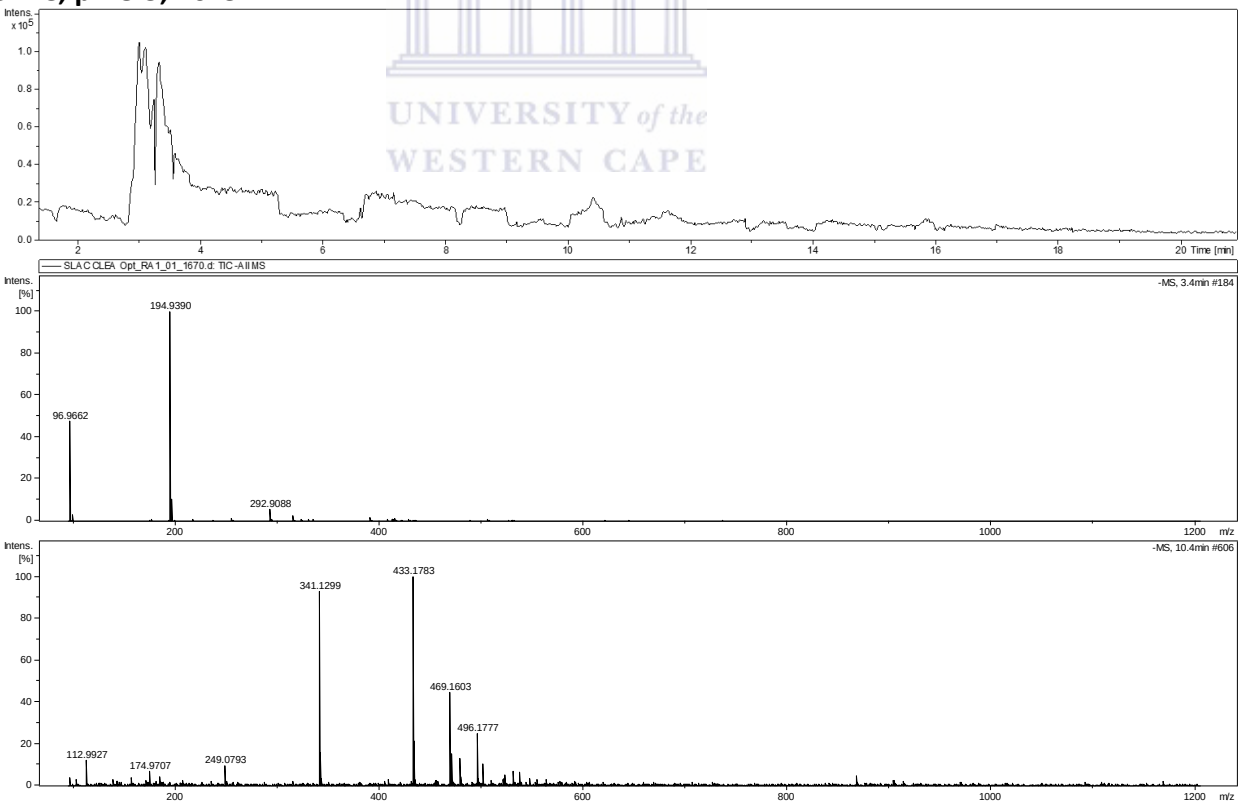
TvL; pH 5.0; 20°C

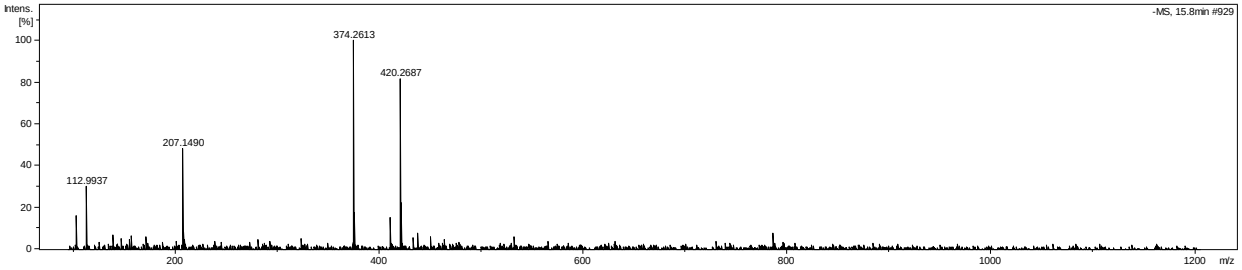


TpL; pH 8.5; 20°C



SLAC; pH 8.5; 20°C





SLAC-VN; pH 7.0; 30°C