ENCAPSULATION OF LACCASE ENZYMATIC EXTRACT, FROM *CORIOLUS HIRSUTUS*, AND ITS BIOCATALYSIS IN SOLVENT-FREE MEDIA

by

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SHORT TITLE

BIOCATALYSIS OF ENCAPSULATED LACCASE IN SOLVENT-FREE MEDIA

This thesis is dedicated to my parents and grandparents, with love.

ABSTRACT

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The optimization of the encapsulation of a laccase enzymatic extract, obtained from *Coriolus hirsutus*, and its biocatalysis in solvent-free media (SFM) for the synthesis of selected oligophenols were investigated. Among the selected matrices, silica sol-gel was found to be the most appropriate one and its use resulted in an enhancement of 319.9% of the residual laccase activity of the encapsulated enzyme as compared to that of the free one as well as by 90% of encapsulation efficiency (EE). In addition, the incorporation of silica into alginate demonstrated a relatively higher EE (70%) as compared to that for the alginate (59%). Initial experimental trials were carried out to screen and to identify the most significant process parameters for the silica sol-gel encapsulation of the laccase enzymatic extract; these parameters included gel drying and aging time, water to silane molar ratio (*r*), hydrochloric acid (HCl) content and protein loading. A central composite rotatable design (CCRD) was used to evaluate the effects of *r*, HCl content and protein loading on the residual laccase enzymatic activity of the sol-gel encapsulated enzyme extract. The experimental findings indicated that *r* and acid content exhibited the most significant effects on the residual laccase activity. Although *r* was the most significant independent variable, it showed a significant opposite interaction effect to that with the acid content. The optimal conditions were for *r* of 6.78, HCl content of 3.22 µmol and protein loading of 0.86 mg/mL sol, which resulted respectively, in the predicted and observed residual laccase activity of the encapsulated enzyme of 445.27 and 443.31% as compared to that of the free one; these findings indicated a valid correlation between the statistical model's predictions and the experimental results. The encapsulated laccase enzymatic extract was also evaluated in terms of its optimum pH and reaction temperature, kinetic parameters, storage stability and re-usability. The results indicated an increase in the enzyme stability and its catalytic efficiency (k_{cat}) of 77.98 min^{-1} for the encapsulated laccase as compared to that of 56.24 min^{-1} for the free enzyme. Using ferulic acid (FA) as a substrate model, the biocatalysis in SFM of the sol-gel encapsulated laccase extract was optimized in terms of enzymatic protein content, reaction temperature and agitation speed; the results showed that the encapsulated enzyme effectively catalyzed the oxidation of FA, where its highest

laccase specific activity (1,309.51 nmol ferulic acid/µg protein/min) was obtained with the use of an optimum protein content of 2 µg, 150 rpm agitation speed and 60°C reaction temperature.

However, there was a complete absence of any laccase activity for the free enzyme in SFM. The encapsulated laccase enzymatic extract also showed a significantly ($P \leq 0.05$) enhanced enzyme specific activity in SFM (238.19 nmol ferulic acid/µg protein/min) as compared to that obtained in the aqueous medium (83.88 nmol ferulic acid/µg protein/min). In addition, the kinetic studies for the biocatalysis of the encapsulated laccase in SFM demonstrated that the enzyme followed the Michaelis-Menten behavior for all selected substrate models, including 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) as well as ferulic, chlorogenic and gallic acids, with K_m value of 0.17, 2.35, 7.67 and 4.62 µmol, respectively, and a corresponding k_{cat} value of 2.84, 4.72, 4.05 and 2.37 min^{-1}. The structural characterization of the end products of FA, obtained by the biocatalysis of encapsulated laccase in SFM, was carried out, using size-exclusion chromatography (SEC), high-pressure performance chromatography (HPLC), spectrophotometric scanning (UV-VIS), Fourier-transform infrared spectroscopy (FTIR) and mass spectrometry (MS). The biocatalysis of the sol-gel encapsulated laccase in SFM resulted in the synthesis of FA oligomers, including its dimers, trimers and tetramers. The FTIR and MS analyses of these oligomers confirmed the oligomerization of FA via C-O-C and C-C linkages. In addition, the laccase-catalyzed end products showed a relative enhancement of the antioxidant capacity with a 1.12 and 1.07-fold increase, respectively, in the 2,2-diphenyl-1-picrylhydrazyl (DDPH) radical scavenging and oxygen-radical absorbance capacity (ORAC) as compared to those for the substrate monomer FA.

RÉSUMÉ

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L'optimisation de la biocatalyse d'un extrait enzymatique de la laccase, obtenu à partir de *Coriolus hirsutus*, et sa biocatalyse en milieu libre de solvent (SFM) pour la synthèse d'oligomères sélectionnés ont été étudiés. Parmi les matrices sélectionnées, le sol-gel de silice s'est avéré le plus approprié et son utilisation menant à une augmentation de 319,9% de l'activité résiduelle de la laccase encapsulée en comparaison de celle de l'extrait libre, et avec une efficacité d'encapsulation (EE) de 90%. De plus, l'incorporation de silice dans l'alginate a démontré une EE (70%) relativement plus élevée que celle pour l'alginate (59%). Des essais expérimentaux ont été réalisés afin de déterminer et d'identifier les paramètres les plus significatifs pour le développement de la procédure d'encapsulation de l'extrait enzymatique de la laccase par le sol gel de silice; ces paramètres incluaient, respectivement, le temps de séchage et de vieillissement de gel de 6 et 24 h, le ratio molaire d'eau au gel (*r*), le contenu en acide hydrochlorique (HCl) et la charge protéique. Une conception composite centrale (CCRD) a été utilisée pour évaluer les effets de *r*, du contenu en HCl et de la charge protéique sur l'activité résiduelle de la laccase encapsulée par le sol gel. Les résultats expérimentaux ont montré que le *r* et le contenu en HCl ont les effets les plus significatifs sur l'activité résiduelle de la laccase. Bien que *r* soit la variable indépendante la plus significative, il a démontré un effet d'interaction opposé à celui du contenu en acide. Les conditions optimales de 6,78 pour le *r*, de 3,32 μmol pour l'HCl et de 0.86 mg pour la charge protéique/mL sol ont mené à 445,27 et 443,31% d'activité résiduelle de la laccase, respectivement, estimée et observée; ces résultats indiquent une bonne corrélation entre les valeurs prédites dans le modèle statistique et celles expérimentales. L'enzyme encapsulée a été aussi évaluée en termes de son pH et température optimales, des cinétiques enzymatiques, de sa stabilité et de sa réutilisation. Les résultats tendent à montrer qu'il y a une augmentation dans la stabilité et l'efficacité catalytique (k_{cat}) de 77,98 min^{-1} pour la laccase encapsulée en comparaison de celle de 56,24 min^{-1} pour l'enzyme libre. En utilisant l'acide férulique (FA) comme substrat modèle, la biocatalyse en milieu de SFM de la laccase encapsulée en sol-gel a été optimisée en termes de la charge protéique, de la température de la réaction enzymatique et de la vitesse d'agitation.

Les résultats ont montré que la laccase encapsulée a effectivement catalysée l'oxydation de FA, où l'activité spécifique de l'enzyme la plus élevée (1309,51 nmol acide férulique/µg protéine/min) a été obtenue avec l'utilisation de 2 µg de protéine, de 150 rpm de vitesse d'agitation et de 60°C de température de la réaction enzymatique. Cependant, il y avait une absence complète de l'activité de la laccase pour l'enzyme libre en milieu SFM. La laccase encapsulée a démontré significativement ($P \leq 0.05$) une activité enzymatique spécifique, en milieu SFM (238.19 nmol acide férulique/µg protéine/min) plus élevée en comparaison à celle obtenue en milieu aqueux (83.88 nmol acide férulique/µg protéine/min). De plus, les études cinétiques pour la biocatalyse en milieu SFM de la laccase encapsulée ont démontré que l'enzyme suit la cinétique de Michaelis-Menten pour tous les substrats modèles, incluant le sel d'ammoniaque de 2,2-azino-bis-(3-ethylbenzothiazoline-6-acide sulfonique (ABTS) ainsi que les acides férulique, chlorogénique et gallique, avec un K_m de 0,17, 2,35, 7,67 and 4,62 µmol, respectivement, et un k_{cat} correspondant de 2,84, 4,72, 4,05 et 2,37 min^{-1}. La caractérisation structurale des produits de FA, obtenue par la biocatalyse de la laccase encapsulée en milieu SFM, a été réalisée par chromatographie sur gel d'exclusion (SEC), chromatographie à haute pression (HPLC, balayage spectrophotométrique (UV-VIS), spectroscopie d'infra-rouge à transformation Fourier (FTIR), et spectroscopie de masse (MS). La biocatalyse en milieu SFM de la laccase encapsulée a mené à la synthèse des oligomères de FA. Les analyses de FTIR et de MS de ces oligomères ont confirmé l'oligomérisation de FA via les liaisons de C-O-C et de C-C. De plus, le produit de la biocatalyse de la laccase a démontré une augmentation relative de sa capacité antioxydante de 1,12 et de 1,07, respectivement, pour le 2,2-diphényl-1-picrylhydrazyl (DDPH) et pour la capacité d'absorbance de l'oxygène radicale (ORAC) en comparaison du monomère FA.

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CONTRIBUTION OF AUTHORS

As per the guidelines of the McGill University Faculty of Graduate Studies and Research "Guidelines for a Manuscript Based Thesis Preparation", the published, submitted and in preparation for submission manuscripts as well as the contribution of the authors are described below.

The principle author, Jagpreet K. Gill, was responsible for the concepts, design of the experimental work and the preparation of the manuscripts as well as the thesis document.

Dr. Valérie Orsat and Dr. Selim Kermasha, the co-authors as well as the thesis supervisor and co-supervisor, respectively, supervised the research work and provided valuable inputs, monitored the progress of the research work and critically reviewed and edited the manuscripts as well as the thesis document, before their submission.

Dr. Salwa Karboune co-authored the third (Chapter 5), fourth (Chapter 6) and fifth (Chapter 7) manuscript, where she provided valuable inputs in the design of the encapsulation trials with the central composite rotatable model as well as the mass spectrometry analysis and also offered the opportunity to use her facilities for carrying out certain experimental work.

Dr. Varoujan Yaylayan, co-author of the fifth manuscript (Chapter 7), contributed to the FTIR analysis and its interpretation for the laccase-catalyzed end products.

The manuscripts included in the thesis are as follows:

1. **Gill, J**., Orsat, V. and Kermasha, S. (2018). Optimization of encapsulation of a microbial laccase enzymatic extract using selected matrices. *Process Biochem.* **65**, 55-61.

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3. **Gill, J.K**., Orsat, V., Karboune, S. and Kermasha, S. (2018). Optimization of silica sol-gel encapsulation of laccase enzymatic extract using response surface methodology. (In preparation for submission)

4. **Gill, J.K**., Orsat, V., Karboune, S. and Kermasha, S. (2018). Biocatalysis of free and silica sol-gel encapsulated laccase extract, from *Coriolus hirsutus*, in solvent-free media. (In preparation for submission)

5. **Gill, J.K**., Orsat, V., Karboune, S., Yaylayan, V. and Kermasha, S. (2018). Characterization of laccase-catalyzed end products, using ferulic acid, in solvent-free media. (In preparation for submission)

CLAIMS OF ORIGINAL RESEARCH

1. This is the first study, where the encapsulation of laccase enzymatic extract obtained from *Coriolus hirsutus*, was optimized using selected matrices including alginate, alginate-silica and silica sol-gel.

2. Screening experiments were carried out for the assessment of selected process parameters for the silica sol-gel encapsulation of a laccase enzymatic extract.

3. Using a central composite rotatable design (CCRD), this is the first time where an efficient method for the silica sol-gel encapsulation of laccase enzymatic extract, was optimized.

4. A novel sol-gel encapsulated laccase enzymatic extract was investigated in terms of its biocatalysis in solvent-free media (SFM), using selected phenolic substrate models.

5. Using ferulic acid as a substrate model, this is the first study to investigate the end products of the sol-gel encapsulated laccase biocatalysis in SFM which were characterized in terms of their structural chemical properties as well as their antioxidant capacity.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAPH	2,2'-azobis-2-methyl-propanimidamide dehydrochloride
ABTS	2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ADH	Alcohol dehydrogenase
Alg-Si M1	Alginate-silica method 1 hybrids
Alg-Si M2	Alginate-silica method 2 hybrids
APCI	Atmospheric pressure chemical ionization
ATCC	American Type Culture Collection
a_w	Water activity
BBD	Box-Behnken design
BSA	Bovine serum albumin
CCD	Central composite design
CCRD	Central composite rotatable design
CLECs	Cross-linked enzyme crystals
C-C	Carbon to carbon coupling bond
C-O	Carbon to oxygen coupling bond
COOH	Carboxyl group
DPPH	2,2-diphenyl-1-picryl hydrazyl
ε	Molar extinction coefficient
EE	Encapsulation efficiency
EPR	Electron paramagnetic resonance
ESI-MS	Electrospray ionization-mass spectrometry
FA	Ferulic acid
FCCD	Face-centered composite design
FRAP	Ferric reducing/antioxidant power
FTIR	Fourier-transform infrared spectroscopy
GC/MS	Gas liquid chromatography/mass spectrometry
(GPC)-HPLC	Gel permeation chromatography-HPLC
HCl	Hydrochloric acid
HOO	peroxyl radicals

HPLC	High-Performance Liquid Chromatography
IC_{50}	Half maximal inhibitory concentration
ICDBs	Inter-domain copper binding sites
k_{cat}	Catalytic efficiency determined as V_{max}/K_m
*k*Da	Kilo Dalton
K_m	Michaelis-Menten constant
λ_{max}	Maximum absorbance
LbL	Layer by layer
LC	Liquid chromatography
MCBPs	Multi copper blue proteins
MCOs	Multi copper oxidases
MS	Mass spectrometry
MW	Molecular weight
NO	Nitric oxide
NOO	Peroxynitrite
ORAC	Oxygen radical absorption capacity
OSM	Organic solvent system
PAHs	Polyaromatic hydrocarbons
R^2	Coefficient of determination
r	Water to silane molar ratio
ROO	alkyl peroxyl radicals
(RP)-HPLC	Reverse phase-high performance liquid chromatography
RSD	Relative standard deviation
RSM	Response surface methodology
SAR	Structure-activity relationship
SD	Standard deviation
SEC	Size-exclusion chromatography
SFM	Solvent-free media
SOLAC	Sol-gel laccase

T-1	Type one copper atom
T-2	Type two copper atom
T-3	Type three copper atom
TEAC	Trolox equivalent antioxidant capacity
TE	Trolox equivalents
TEOS	Tetraethyl orthosilicate
THF	Tetrahydrofuran
TMS	Ternary micellar system
TRAP	Total radical absorption potentials
V_{max}	Maximum reaction rate

CHAPTER I

INTRODUCTION

In recent years, antioxidants have become a more and more essential group of food additives and this is due to their ability to enhance the nutritional quality and for their capacity of extending the shelf-life (Shahidi and Ambigaipalan, 2015). Antioxidants are also incorporated in food products for better preservation of their natural color and flavor (Shah *et al*., 2014). In general, the use of antioxidants in food systems requires them to be inexpensive, non-toxic, effective at low concentrations, stable, odorless, tasteless, colorless and they should have a good solubility (Shahidi and Ambigaipalan, 2015). The most common synthetic antioxidants in food industry are butylated hydroxyanisole, butylated hydroxytoluene, ter-butyl hydroquinone and propyl gallate (Cömert and Gökmen, 2018). However, there has been a growing concern over the use of these synthetic antioxidants in terms of their potential toxicity, high manufacturing costs and lower efficiency which has created a need for alternative natural sources of food antioxidants. Plant tissues are known to be natural sources of various antioxidants such as, tocopherols, ascorbic acid, carotenoids and phenolic compounds (Cömert and Gökmen, 2018).

Phenolic compounds are plant secondary metabolite derivatives with antioxidant capacity; they have been associated with several health benefits, such as anti-allergic, anti-thrombic, anti-inflammatory, cardioprotective, vasodilatory and antioxidant effects (Balasundram *et al.*, 2006). Polyphenols are ubiquitous in all plants and hence they are major components of the human diet (Haminiuk *et al*., 2012). The structure of phenolic compounds is the key determinant for their free radical scavenging as well as for their metal chelating capacity and is referred to as their structure-activity relationship (SAR); however, their heterogeneity, large molecular weights and the increasing extent of their polymerization has raised many difficulties in their structural analyses (Rice-Evans *et al.*, 1996).

On the other hand, it has been reported that phenolic oligomers, including dimers, trimers and tetramers, demonstrated improved physiochemical properties, such as a higher absorption and lower non-metal or protein complexation in comparison to their respective polymers (Desentis-Mendoza *et al.*, 2006; Lotito *et al*., 2006). Oligophenols have also been reported to show enhanced anti-inflammatory and anti-bacterial effects (Aruoma *et al.*, 2006; Adelakun *et al.*, 2012a). The

oligomerization of phenolic compounds could result in novel biomolecules of higher antioxidant capacity, where the synthesis of such oligophenols may be carried out through chemical or enzymatic polymerization of monomers or the de-polymerization of polymers (Bourbonnais *et al.*, 1995; Nutsubidze *et al.*, 1998; Sanchez-Cortes *et al.*, 2001; Desentis-Mendoza *et al.*, 2006; Reihmann and Ritter, 2006; Adelakun *et al.*, 2012a). Enzyme-catalyzed syntheses offer several advantages over chemical ones as they show higher substrate specificity and can be carried out under relatively mild conditions of temperature, pH, pressure and without the use of toxic solvents (Klibanov, 2001; Desentis-Mendoza *et al.*, 2006).

Laccases (EC 1.10.3.2) are multi-copper oxidases that have gained increasing interest as a result of their broad substrate specificity, high catalytic rate and their ability to use the environmental oxygen as co-factor (Jaiswal *et al*., 2014). In nature, laccases are found in several species of fungi, bacteria as well as higher plants and they have been associated with delignification, pigment formation and detoxification (Strong and Claus, 2011). The wide range of substrate specificity of laccase has resulted in many industrial applications in food processing, textile, pulp and paper, pharmaceutical, cosmetics, biosensors, bioremediation, bio-bleaching, detoxification of dyes and treatment of waste waters (Yamak *et al.*, 2009; Strong and Claus, 2011).

Research work, carried out in our laboratory, has succeeded in the laccase-catalyzed synthesis of selected oligophenols in aqueous and organic solvent media (OSM) (Bou-Mitri, 2013). In general, aqueous solutions are the most frequently used media for carrying out the enzyme-catalyzed reactions; however, the synthesis of oligophenols has been reported to result into unstable oligomers often leading to the formation of higher phenolic polymers. The instability of synthesized oligomers, formed in the aqueous medium, has been reported to be reduced in non-conventional organic solvents (Kazandjian and Klibanov, 1985; Mustafa *et al.*, 2005; Bou-Mitri, 2013). Although the use of organic solvents leads to the formation of stable oligophenols, the use of hazardous chemicals in large-scale industrial processes poses great concern to the product safety and to the environment. Alternatively, reactions carried out in solvent-free media (SFM) could overcome such limitations, in addition to which, these reactions offer reduced reaction times, cost efficiency and lower energy consumptions (Walsh *et al.*, 2007; Aziz *et al*., 2012; Sorour *et al*., 2012a,b).

The biocatalysis of enzymes in non-conventional media is rather limited, due to their sensitivity to the surrounding environment. In order to overcome such limitations, the enzymes could be immobilized, possibly resulting in more stable and effective biocatalysts (Fernández-Fernández *et al*., 2013). The encapsulation of enzymes is the most appropriate approach for their immobilization as it avoids any detrimental changes to their native structure (Daâssi *et al*., 2014). Among the various matrices used for the encapsulation of laccase, alginate is one of the most commonly employed biopolymers, because of its mild gelling properties and its non-toxicity; however, the encapsulation of enzymes in alginate gels is mostly associated with a lower stability and an uncontrolled porosity (Coradin *et al*., 2003). In order to develop more robust matrices, the incorporation of silica into alginate has been investigated for the encapsulation of biomolecules (Meunier *et al*., 2010). The encapsulation of enzymes in silica sol-gel matrices offer several other advantages, including non-toxicity, improved enzyme stability, optical transparency, enhanced re-usability and storage stability as well as the possibility of tuning the gel porosity (Vila-Real *et al*., 2010; Lloret *et al*., 2011). Hence, the encapsulation of the laccase enzymatic extract in silica-sol gel could be an effective method and may result in its enhanced stability, re-usability and catalytic activity. Mohidem and Mat (2012a) reported that the encapsulation of laccase in inorganic silica sol-gels has been related to an enhancement of its catalytic efficiency.

In order to investigate the independent and mutual interaction effects of selected process parameters for the encapsulation of laccase enzymatic extract, the response surface methodology (RSM) could be considered as an efficient statistical technique (Myers *et al*., 2009). The central composite design (CCD), the face-centered composite design (FCCD) and the central rotatable composite design (CRCD) are the most known second-order models that are used in RSM and are classified according to their required number of experiments (Paulo and Santos, 2017). On the other hand, the Box-Behnken design (BBD) could also be used as it offers fewer experimental trials as compared to the other RSM designs, using the same number of factors (Mansor *et al*., 2016). The optimization of the immobilization of laccase has been recently reported, using the BBD (Mansor *et al*., 2016; Vera and Rivas, 2017).

Several methods have been reported for the characterization of the laccase-catalyzed end products. Size-exclusion chromatography (SEC) has been widely reported for the separation of the enzyme-catalyzed phenolic polymers (Bou-Mitri, 2013; Jiang *et al*., 2017). On the other hand, high-

performance liquid chromatography (HPLC) is the most widely used separation and analytical technique for the characterization and quantification of phenolic compounds (Pyrzynska and Sentkowska, 2015). Fourier-transform infrared (FTIR) is commonly used for the determination of the polymerization reactions and the coupling modes (Bozic *et al*., 2012). In addition, mass spectrometry (MS) has also been widely used for the characterization of polymers and their structural determination (Bou-Mitri, 2013).

The antioxidant capacity of laccase-catalyzed oligophenols could be carried out by the oxygen radical absorption capacity (ORAC) and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay (Karadag *et al*., 2009). ORAC is one of the most widely used antioxidant assays and could imitate the model reactions of the antioxidant interactions with lipids in food and physiological systems; moreover, this method can identify both hydrophilic and hydrophobic antioxidants by altering the solvent and the radical sources (Adelakun *et al*., 2012a,b). Alternatively, for the DPPH method the free radical scavenging capacity is measured by following the absorbance decrease at 515 nm, where the relative percentage of residual DPPH is considered directly proportional to the antioxidant concentration; this method is known for its simplicity and reduced analysis time (Prior *et al*., 2005; Karadag *et al*., 2009).

The overall objective of this research work was to optimize the encapsulation of the laccase enzymatic extract, from *Coriolus hirsutus*, as well as its biocatalysis in SFM, for the synthesis of selected oligophenols of higher antioxidant capacity. The proposed research was also aimed at the structural characterization and the determination of the antioxidant capacity of the synthesized end products.

The specific objectives of the research were:

(i) To investigate the optimization of encapsulation of the laccase enzymatic extract, obtained from *Coriolus hirsutus*, in selected matrices, including alginate, alginate-silica and silica sol-gel.

(ii) To screen and investigate the effects of selected process parameters, to identify the most significant ones that have an impact on the silica sol-gel encapsulation of the laccase enzymatic extract.

(iii) To optimize the silica sol-gel encapsulation of laccase enzymatic extract, using response surface methodology (RSM).

(iv) To optimize the biocatalysis of the silica sol-gel encapsulated laccase extract in SFM.

(v) To characterize the sol-gel encapsulated laccase synthesized end products in SFM, in terms of their chemcial structure and antioxidant capacity.

The present thesis document consists of seven chapters. Chapter I provides a general introduction, whereas Chapter II covers the literature review of the relevant topics and concepts that are used to undertake the research work. Chapter III describes the encapsulation of the laccase enzymatic extract, from *C. hirsutus*, in selected matrices, that were assessed in terms of their encapsulation efficiency (EE) and residual laccase enzymatic activity. In addition to this, the encapsulated laccase was characterized for its kinetic parameters, protein leakage, re-usability and storage stability. Chapter IV describes the screening of the selected process parameters for the optimization of the encapsulation of the laccase enzymatic extract in silica sol-gel. Chapter V reports on the optimization of the silica sol-gel encapsulation of the laccase enzymatic extract in silica sol-gel matrix, using RSM. Chapter VI deals with the optimization of the biocatalysis of the sol-gel encapsulated laccase extract, in SFM. Chapter VII covers the characterization of the sol-gel encapsulated laccase-catalyzed end products, synthesized in SFM, in terms of their structural characteristics and antioxidant capacity.

CHAPTER II

LITERATURE REVIEW

2.1. Phenolic compounds

Phenolic compounds are secondary metabolite derivatives produced from the pentose phosphate, shikimate and phenylpropanoid pathways in plants (Balasundram *et al.,* 2006). These derivatives are produced during normal plant metabolism and are synthesized in response to stress conditions such as disease, wounding, temperature, humidity and UV radiations (Naczk and Shahidi, 2004). Phenolic compounds display an active role in plant physiology and are essential for the growth and development of all plant species; these bioactive compounds may serve as phytolexins, attractants for pollination, anti-feedants, antioxidants and are also involved in the plant pigmentation process (Balasundaram *et al*., 2006; Ignat *et al*., 2011).

2.1.1. Structure and classification

The general structure of phenolic compounds consists of an aromatic ring bearing one or more hydroxyl groups or substituents and may range from simple phenolic molecules to highly polymerized forms. Phenolic compounds display a wide structural diversity and have been classified according to their basic aromatic ring and side chain arrangements; however, they are grouped together as compounds that are commonly known as 'polyphenols' (Balasundram *et al.,* 2006). Polyphenols are classified into various classes based on their number of phenol rings and structural elements that bind these rings together (Table 2.1) (Manach *et al.,* 2004). There are more than 8000 known structures of polyphenols, amongst which, flavonoids contribute over 4000 structures (Harborne and Williamson, 2000; Cheynier, 2005). Out of the several classes of polyphenols, phenolic acids, tannins and flavonoids are abundant in our diet (Balasundram *et al*., 2006).

2.1.2. Biological properties

Phenolic compounds have been reported for their wide range of physiological properties that demonstrate major health effects. Several literature studies have associated them with varied health benefits, including anti-allergic, anti-thrombic, anti-inflammatory, cardioprotective, vasodilatory and antioxidant effects (Samman *et al*., 1998; Balasundram *et al.,* 2006). The antioxidant capacity

of polyphenols is due to their ability to chelate several metal ions and scavenge free radical species such as peroxyl radicals (HOO), alkyl peroxyl radicals (ROO), nitric oxide (NO) and peroxynitrite (NOO). In nature, phenolic compounds have demonstrated this antioxidant capacity and prevented the oxidation of macromolecules such as DNA, lipids, proteins and nucleic acids in biological systems (Haminiuk *et al*., 2012).

2.2. Oligophenols

2.2.1. Structure

Oligophenols are the condensed form of phenolic monomers and consist of low molecular weight compounds ranging from dimers, trimers up to octamers. They occur in plants as intermediate compounds synthesized via the condensation reaction of polymers during enzymatic browning or lignin and tannin formation (Shahidi and Naczk, 1995; Bou-Mitri, 2013). The oligomers of hydrolysable tannins have been widely reported in medicinal plants containing variable structures such as; gamins A, B and C isolated from *Geum japonicum* and rugosins D, E and F obtained from *Rosa rugosa*, both of which are anti-inflammatory and anti-diarrheic medicinal plants (Okuda, 2005).

The oligomers reported in literature are mostly of flavonoid and phenolic acids (Bravo, 1998; Handique and Baruah, 2002; Cheynier 2005; Okuda, 2005; Fraga, 2007; Bunzel, 2010; Rockenbach *et al*., 2011; Bozic *et al*., 2012; Chen *et al*., 2016). The chemical structures of oligophenols are not defined by the types of their initial monomers, but by the linkage existing between them. Various plants demonstrate their unique pattern of oligomerization, where their pattern differs with the species e.g., most procyanidins found in cocoa are B-type oligomers (Fraga, 2007). The oligomers of phenolic acids are not well described in literature, except for the oligomers of hydroxycinnamic acids as those reported by Handique and Baruah (2002) (Fig. 2.2). Hydroxycinnamic acid derivatives are the condensation oligomers of mono-lignols including coniferyl, *p*-coumaryl and sinapyl alcohol (Handique and Baruah, 2002). The ferulic acid dimers were first identified 40 years ago and ferulate oligomers have also been extracted from plants (Bunzel, 2010). Bou-Mitri (2013) reported on the laccase catalyzed oligomers of ferulic acid that were synthesized in aqueous and organic solvent media (OSM).

Table 2.1. General classification of phenolic compounds (Balasundram *et al*., 2006; Bou-Mitri, 2013).

Basic skeleton	Class	Example
C_6	Simple Phenolics Benzoquinones	Phenol, cresol, thymol, resorcinol
C_6-C_1	Phenolic acids	Gallic, syringic, vanilic, *p*-hydroxybenzoic acid
C_6-C_2	Acetophenones Phenyl acetic acids	
C_6-C_3	Hydroxycinnamic acids Phenylpropanoids	Synapic, ferulic, *p*-coumaric, caffeic acid Coumarins, isocoumarins, chromenes
C_6-C_4	Napthoquinones	Juglone
C_6-C_1-C_6	Xanthones	Mangiferin
C_6-C_2-C_6	Stilbenes Anthraquinones	Resveratrol
C_6-C_3-C_6	Flavanoids Isoflavanoids	Quercetin, catechin, naringenin Diadzein, genistein, glycitein
$(C_6$-$C_3)_2$	Lignans, neolignans	Sesamin
$(C_6$-C_3-$C_6)_2$	Bioflavanoids	
$(C_6$-$C_3)_n$	Lignins	
$(C_6$-C_3-$C_6)_n$	Condensed Tannins	Procyanadin

2.2.2. Potential health effects of oligophenols

The heterogeneity of polyphenols provides difficulties in their analysis due to their large molecular weights and increasing extent of polymerization, hence most studies consider monomers and low molecular weight oligomers (Cheynier, 2005). Phenolic oligomers are associated with several biological benefits. In comparison to high molecular weight polymers, the oligomerization of phenolic compounds has been linked to an increase in their absorption and bioavailability which may be due to an improvement of their physico-chemical properties and lower or non metal as well as protein complexation (Natella *et al.,* 1999; Lu *et al.,* 2006a).

Oligophenols have also been studied for their antitumor effects. Barjot *et al*. (2007) investigated the effect of resveratrol as compared to two of its oligomers and the results showed that the oligomer Miyabenol C resulted in maximum inhibition of cell proliferation. Gescher *et al*. (2011) investigated the antiviral activity of oligomeric and polymeric procyanidins, where the antiviral activity was observed only for the oligomeric forms.

The effect of oligomer chain length was evaluated by Lotito *et al.* (2006), where it was reported that the antioxidant capacity of procyanidins varied significantly with the oligomer chain length; these authors (Lotito *et al*., 2006) suggested that the monomers, dimers and trimers of catechin were found to be more effective antioxidants against liposome oxidation in aqueous phase whereas, higher polymers were effective in lipid domains. Desentis-Mendoza *et al.* (2006) found that the low molecular weight aggregates of quercitin demonstrated a higher antioxidant capacity as compared to their respective monomers. Adelakun *et al*. (2012a) reported that the dimerization of FA led to the amplification of its antioxidant capacity. Likewise, Bou-Mitri (2013) indicated that the FA dimer obtained by laccase-catalyzed synthesis in OSM, demonstrated the highest antioxidant capacity as compared to that for the FA and the end-products obtained in the aqueous medium.

In general, the potential health effects of phenolic compounds and their bioavailability depends on their metabolism and absorption in the body, which majorly depends on their molecular weight and degree of glycosylation/acylation. Bravo (1998) described the role of the solubility of polyphenols in their absorption, where the presence of non-extractable polyphenols that were recovered in the faeces indicated their resistance to intestinal digestion and/or absorption.

However, the author (Bravo, 1998) reported an absence of oligophenols in the faeces which confirmed their enhanced absorption in the gastrointestinal tract as compared to the higher degree polymers.

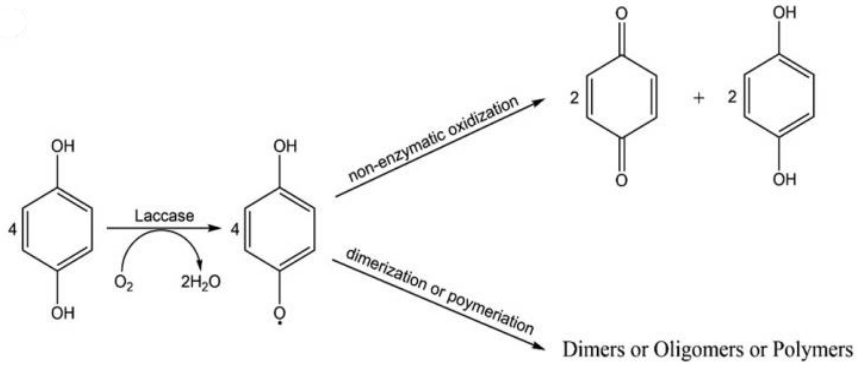


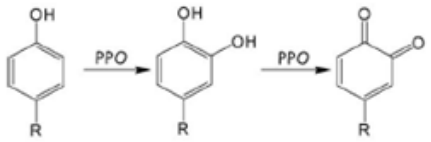
Figure 2.1. Laccase-catalyzed oxidation of hydroquinone (Su *et al*., 2018).

2.2.3. Synthesis of oligophenols

2.2.3.1. Enzymatic polymerization/de-polymerization reaction

Phenolic oligomers possess various beneficial effects and enhanced properties in comparison to their respective polymers. The synthesis of phenolic oligomers may be carried out chemically or through enzymatic polymerization and de-polymerization pathways (Fig. 2.1). The enzyme-catalyzed polymerization of phenols is conducted in three subsequent steps; initiation, propagation and termination. The polymerization reaction is initiated by a polyphenol oxidase or peroxidase enzyme resulting in the generation of a free radical from the phenolic compound and a phenoxy radical in case of phenols (De Beer *et al*., 2002; Reihmann and Ritter, 2006). The initiation reaction is the only step controlled by enzyme kinetics; the phenoxy radicals or quinones are highly reactive species and easily attack other monomers to form low molecular weight oligomers or polymers via successive recombination and radical transfer steps. The reaction may be terminated by reaction of free radicals amongst themselves or with antioxidants resulting in the formation of inert end products (Reihmann and Ritter, 2006).

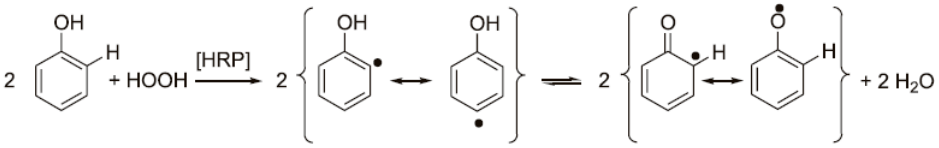
The overall polymerization may be depicted as follows:



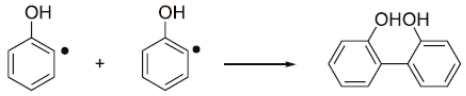
(1)

$$2PhOH + H_2O_2 \rightarrow 2PhO^{\bullet} + 2H_2O \qquad (2)$$

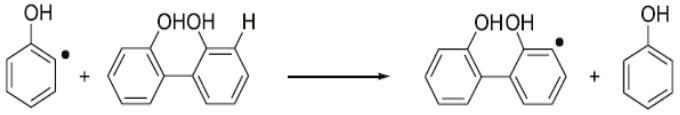
The following reactions describe the mechanism of phenolic polymerization catalyzed by horseradish peroxidase in the presence of hydrogen peroxide and could also be carried out in the presence of polyphenol oxidases, that use molecular oxygen instead.



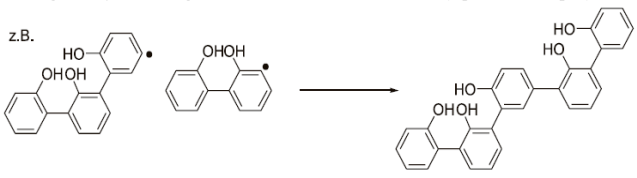
Step 1: Initiation of peroxyl radical formation



Step 2: Conjugation



Step 3: Propagation



Step 4: Polymerization by alternating radical transfer and recombination

Likewise, the de-polymerization reactions are similar to those of the polymerization, except that they occur in the reverse direction. There is a continuous equilibrium between polymerization/de-polymerization reactions and only when the concentration of phenoxy radical decreases below a certain threshold, can the de-polymerization be compared to the polymerization (Reihmann and Ritter, 2006). Nutsubidize *et al*. (1998) reported on lignin de-polymerization and (re) polymerization in white rot fungal media and indicated that extracellular culture solutions tend to exhibit polymerization reactions *in vitro*, while the de-polymerization ones predominate *in vivo*.

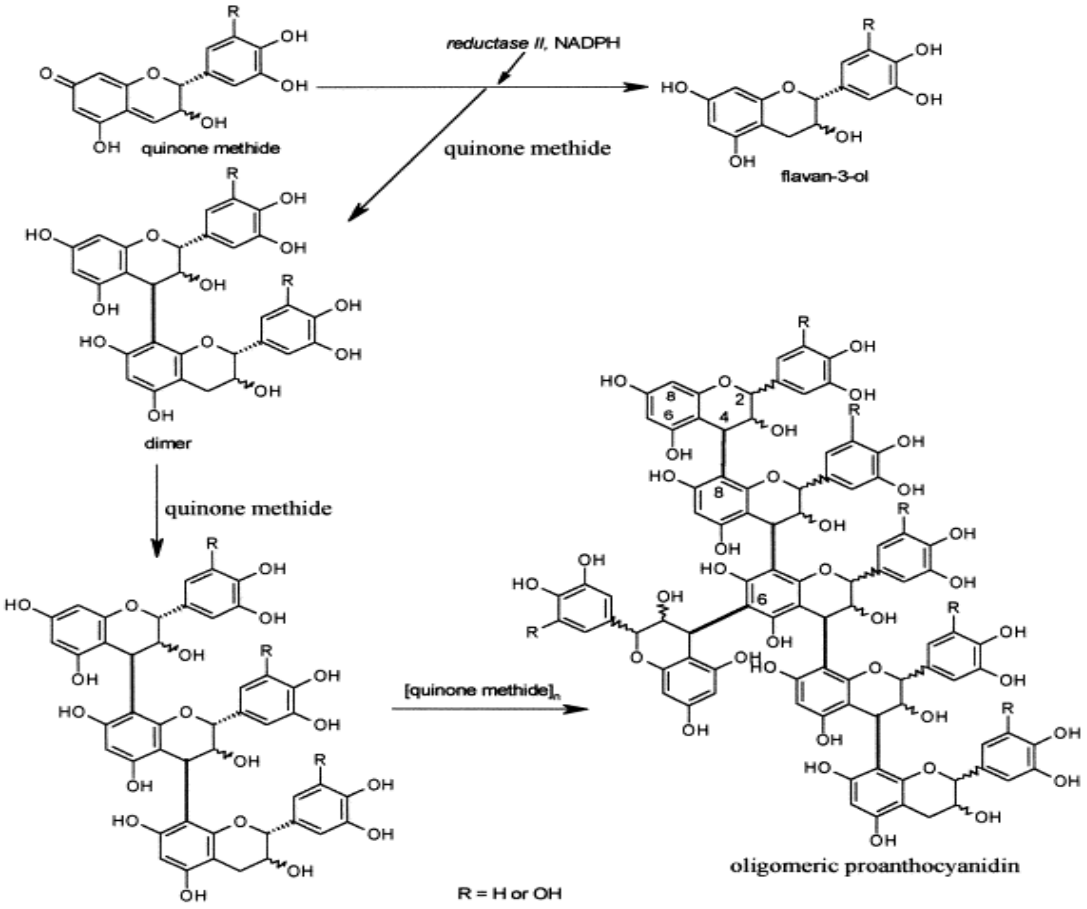


Figure 2.2. Condensation reaction of flavan-3-ol (Handique and Baruah, 2002).

2.3. Laccase

2.3.1. Generality

Laccases (EC 1.10.3.2) belong to a group of polyphenol oxidases, known at multicopper oxidases. Multi copper oxidases (MCOs) or multi copper blue proteins (MCBPs) are further classified into

three sub-groups based on their structural domain functional properties; nitrite reductase types containing two domains, laccase type with three domains and the cerruloplasmin type consisting of six domains. The laccase type and cerruloplasmin types are classified under one group known as MCOs (Nakamura and Go, 2005; Strong and Claus, 2011). The first laccase activity was discovered by Yoshida (1883) in a Japanese laquer tree, *Rhus vernicifera.* By function, laccases are oxidoreductases (Fig. 2.3) and utilize the redox property of the copper ions to catalyze the oxidation of various aromatic (phenols) as well as non-aromatic compounds in the presence of mediators, by the simultaneous reduction of oxygen to water (Mayer and Staples, 2002; Morozova *et al.,* 2007). Even though laccases are characterized by low substrate specificity, they possess an ability to catalyze a wide range of substrates due to their affinity for ortho, para and meta di-phenol groups (Duran *et al.,* 2002).

2.3.2. Sources of laccase

Laccases are ubiquitous in nature and are widely distributed in higher plants, fungi as well as bacterial species. In addition to this, they have also been detected in insects including, *Drosophila, Bombyx, Calliphora* etc. (Fernández-Fernández *et al.,* 2013). The distribution of laccases is most abundant in fungi as compared to plants or bacteria. The wood-rotting fungi are known to be the most common laccase producers, including *Trametes versicolor, Trametes hirsuta, Trametes ochracea, Trametes gallica, Coriolopsis polyzona* and *Cerrena maxima* (Fernández-Fernández *et al.,* 2013). Most fungal laccases are extracellular proteins due to their participation in lignin breakdown; however, intracellularly located laccases have also been reported in some fungal species (Baldrian, 2006; Piscitelli *et al.,* 2010). The report of laccase in higher plants is rather limited which may be due their occurrence as cell wall-bound intracellular enzymes (Mayer and Staples, 2002; Morozova *et al.,* 2007). The first reported presence of plant laccase was in the resin ducts of *Rhus vernicifera*. The presence of laccase activity in bacterial species was first discovered in *Azospirrilum lipoferum* (Givaudan *et al.,* 1993); they have also been reported in *Streptomyces griseus, E.coli, Streptomyces lavendulae* and *Bacillus subtilis* which demonstrate their exceptional versatility (Claus, 2004).

2.3.3. Biochemical structure of laccase

Laccases are monomeric glycoproteins with molecular weights ranging from 50-130 kDa; the extent of glycosylation varies among different sources which contributes to 45% of molecular

weight in plants and up to 10-30% in fungi. Fungal laccases have an isoelectric point (pI) ranging from 3-7 and an optimum pH of 3.6-5.4; whereas, plant laccases have pI ranging from 3-9 with an optimum pH of 6.8-7.4 (Madhavi and Lele, 2009).

In general, the copper atoms present in the enzyme are classified into three kinds; type-1 (T-1), type-2 (T-2) and type-3 (T-3). The laccase type MCBPs consist of four copper domains that are coordinated and classified as per their spectroscopic and electron paramagnetic resonance (EPR) properties. The T-1 (blue) and T-3 copper in laccase produce a maximum absorbance at 610 nm and 330 nm, respectively. The T-1 and T-2 copper are mononuclear copper centers and are EPR detectable; whereas, the T-3 copper center is binuclear and the two copper ions are anti-ferromagnetically coupled via a hydroxyl group bridge (Fig. 2.4). The diamagnetic property of the T-3 copper ions makes them EPR undetectable (Morozova *et al.,* 2007). Furthermore, the T-1 copper consists of two histidines with one cysteine and methionine residue; the first three residues of the T-1 copper form a strong trigonal coordination with the copper ion and is essential for the binding site. The fourth residue methionine is rather weakly coordinated and may be replaced by other amino acid residues such as leucine and phenylalanine (Fig. 2.5). Typically, MCBPs also contain inter-domain copper binding sites (ICDBs) within the main domains containing only histidine residues. The interdomains vary with the type of MCBPs and are mononuclear for the nitrite reductase type and trinuclear in the laccase and cerruloplasmin type. The trinuclear interdomain site in laccase consists of one T-2 copper ion coordinated with two of T-3 copper ions by eight histidine residues (four from each domain) (Morozova *et al.*, 2007).

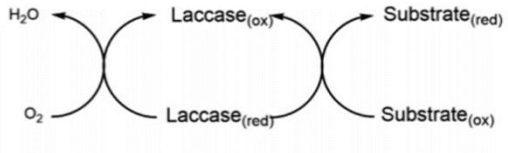


Figure 2.3. Laccase-catalyzed substrate redox cycles (Su *et al*., 2018)

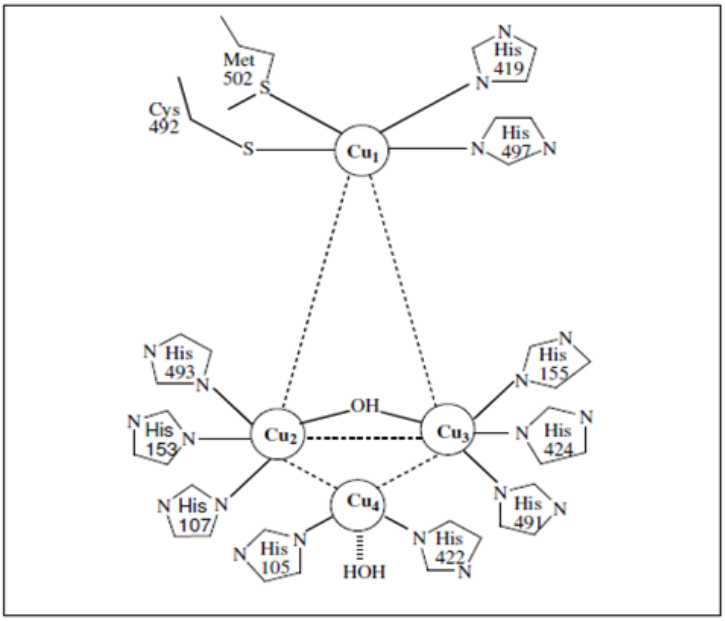


Figure 2.4. Active sites of laccase (Claus, 2004).

2.3.4. Laccase catalysis

Laccase catalytic action involves the blue copper accepting an electron from the substrate (oxidation) that is donated to another substrate by the inter-domain copper ions (reduction). Laccases catalyze the oxidation of several substrates such as phenols, methoxyphenols, aromatic amines, polyaromatic compounds and metals ions which are in the vicinity of the blue copper ion. The electron accepted from the substrate is donated by the inter-domain copper to molecular oxygen, which is converted to water (Nakamura and Go, 2005). The four single electron oxidations takes place in three main steps; the reduction of T-1 copper by a reducing substrate, electron transfer to the T-2 and T-3 copper forming the trinuclear complex and the reduction of oxygen to water at the inter-domain site (Gianfreda *et al.*, 1999). It has been observed that 40% of T-1 and T-3 coppers of the native laccase react with the dioxygen and hence it is the T-2 copper that is involved in the reduction of the dioxygen molecule in the absence of the T-1 copper. Therefore, the type-2/3 trinuclear complex is the active site for the reduction of molecular oxygen and the T-1 copper is not required for reactivity with it (Duran *et al.*, 2002).

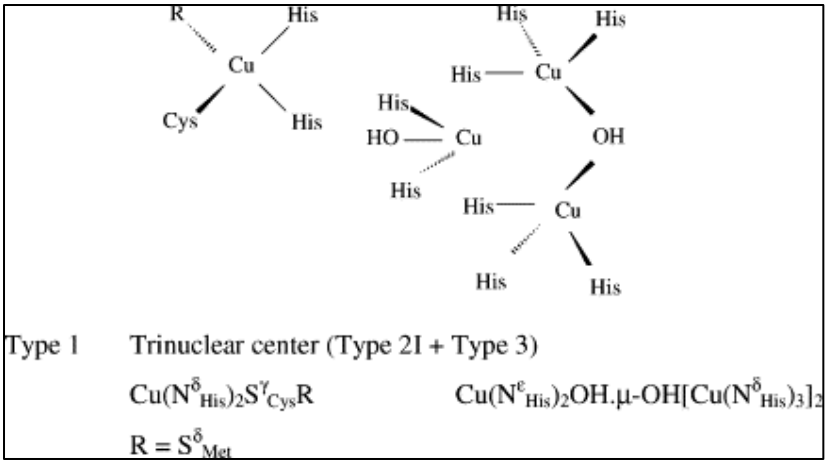


Figure 2.5. Active copper binding sites of laccase (Duran *et al.,* 2002).

2.3.5. Applications of laccase

Laccases form an attractive choice for industrial applications due to their ability to catalyze a wide variety of natural substrates. In nature, fungal laccases have been associated with three major functions; delignification, pigment formation and detoxification (Solomon *et al*., 1996). Their applicability has been proposed in various applications, including food, paper and pulp, textiles, bio-bleaching, bio-remediation, cosmetics, nano-biotech and pharmaceuticals (Piscitelli *et al*., 2010). Potential industrial and biotechnological applications of laccase have been extensively reviewed by Rodriquez Couto and Toco Herrera (2006). Despite the various potential applications of laccase, an efficient use of the enzyme necessitates the development of low cost production processes and immobilization techniques.

2.3.5.1. Laccase applications in food industry

There are numerous laccase substrates naturally found in food and biocatalytic conversion of these substrates is carried out to improve their function, quality and cost reduction. The ability of laccases to enhance or modify the colour of some foods makes them an appropriate choice to be used in the beverage industry, to remove undesirable phenolic compounds which may cause browning, haze and turbidity formation in juices, wines or beers. They have also found an application in the baking sector due to their cross-linking property. Selinheimo *et al*. (2006) reported the increase in resistance of flour dough by use of laccase and also observed a decrease in extensibility of gluten and flour dough. They may also have potential applications in bio-

remediation, biosensors, beverage processing and ascorbic acid formation (Rodriquez Couto and Toco Herrera, 2006). However, these applications also propose the need for low cost efficient enzyme production processes and immobilization techniques for their effective use.

2.3.5.2. Laccase applications in the pulp and paper industry

There are major environmental concerns regarding the conventional chlorine based methods for delignification and bleaching procedures used in the pulp and paper industry. The preparation of paper requires delignification in the wood pulp and justifies proposing laccase as a natural method for the process. Laccase has been found to be a better choice than lignin peroxidase and manganese-dependent peroxidase due to their availability and ease of manipulation (Call and Mucke, 1997). Developed laccase mediator systems developed have been used for delignification of kraft pulps (Cañas and Camarero, 2010).

2.3.5.3. Laccase applications in the textile industry

The textile industry comsumes two thirds of the total dye chemicals available in the market and this is principally used in the wet processing of textiles, thus leading to contaminated waste waters. Most dyes are made from carcinogenic compounds, such as benzidine, as well as some other aromatic compounds. Most of the conventional treatments of dye waste water are not efficient or economical, while it appears that the use of laccases seems an economical and an effective solution to the problem. There are numerous studies involving the use of free laccase and their highly efficient immobilized forms for the treatment and degradation of these synthetic dyes (Rodriquez Couto and Toco Herrera, 2006).

2.3.5.4. Laccase applications in biotechnology

The application of bioelectrochemistry has gained interest in its use for biosensors or detector systems in various analytical techniques. Laccases have been used to detect various phenolic compounds and azides in biosensors, considering their ability to catalyze the electron transfer reactions without the use of cofactors. The immobilization of laccases has been known to have a substantial effect on biosensors and may be used to develop multi-functional biosensors. (Rodriquez Couto and Toco Herrera, 2006).

2.3.5.5. Other applications of laccase

Polyaromatic hydrocarbons (PAHs) and xenobiotics are major sources of soil contamination and catalytic property of laccases may be used to degrade some of these contaminating compounds. This degradation capacity forms the major part of application of laccases in soil bioremediation processes. Laccases may also have potential applications in synthetic chemistry where they may produce complex polymers with protective effects and medicinal uses. Applications in cosmetics is not a novel finding for laccases and they have long been used to produce less-irritant hair dyes, where they replace the conventional use of hydrogen peroxide as the oxidizing agent in the dye formulation (Rodriquez Couto and Toco Herrera, 2006).

2.4. Immobilization of enzymes

2.4.1. General immobilization

Enzyme immobilization is a technique to improve and develop the reusability and stability of enzymes for their application in biocatalytic processes (Ammann *et al.,* 2014). This technique is used for the attachment of free or soluble enzymes to a range of supports resulting in their confinement in the selected matrix (Khan and Alzohairy, 2010). The immobilization of enzymes principally involves the interaction of two components; the enzyme and the carrier support. Hence, the design process of the immobilized enzyme must take into consideration the surface properties of both the enzyme as well as the support matrix. Structurally, enzymes are characterized by polar and non-polar groups; this property may be used for the development of the carrier supports that are modified according to the catalyst (Hanefeld *et al.,* 2009).

The enzyme immobilization techniques (Fig. 2.6) are generally categorized into five types including, adsorption or deposition, entrapment, covalent binding, self-immobilization and encapsulation (Brady and Jordaan, 2009; Hanefeld *et al.,* 2009). The selection of a specific immobilization method may vary for different enzymes; however, the main goal of immobilization is to attain high specific enzyme activities without trading-off the associated advantages. In addition, the use of a specific immobilization matrix should also consider the nature of the reaction media for biocatalysis and must provide better accessibility of the substrate with the immobilized enzyme (Hanefeld *et al.,* 2009). The different reported studies on laccase immobilization are summarized in Table 2.2 and 2.3.

2.4.2. Methods of enzyme immobilization

2.4.2.1. Entrapment

The entrapment of enzymes confines them in a three-dimensional lattice network of a porous matrix which is synthesized by the polymerization of an initial monomer using a cross-linker. The entrapment matrix could be organic (alginate, gelatin, chitin, etc.) or inorganic (silica gel) in nature. The advantage of using entrapment as a method of immobilization is that it is a simple technique and there is no conformational change on the native structure of the enzyme molecule (Fernández-Fernández *et al.,* 2013). The major limitations associated with this method are that of mass-transfer and lower enzyme-loadings (Brady and Jordaan, 2009).

2.4.2.2. Adsorption

The immobilization of enzymes using adsorption may be carried out via ionic interactions or other weak forces of attraction such as van der Waals forces. van der Waals forces or hydrophobic interactions require the presence of large lipophilic surfaces on the enzymes where, they are immobilized on a hydrophobic carrier support. The major limitation of adsorption is the leaching of the immobilized enzyme in aqueous medium. In general, hydrophobic interactions are very weak binding forces and could result in the leakage of enzyme from the support during washing steps (Hanefeld *et al.,* 2009).

2.4.2.3. Covalent binding

Covalent binding is an irreversible method of immobilization, where the enzyme is linked to the support matrix through covalent bonds; the functional groups present on the carrier support are activated and interact with the nucleophilic residues on the enzyme to be immobilized (Fernández-Fernández *et al.,* 2013). The immobilization via covalent bonds minimizes the problem of leaching as the enzyme is tightly linked to the carrier through multiple bonds hence, reducing its conformational flexibility. On the other hand, the method could result in conformational changes to the active site of the native enzyme structure, thereby affecting their specific activity. (Mateo *et al.,* 2007; Hanefeld *et al.,* 2009).

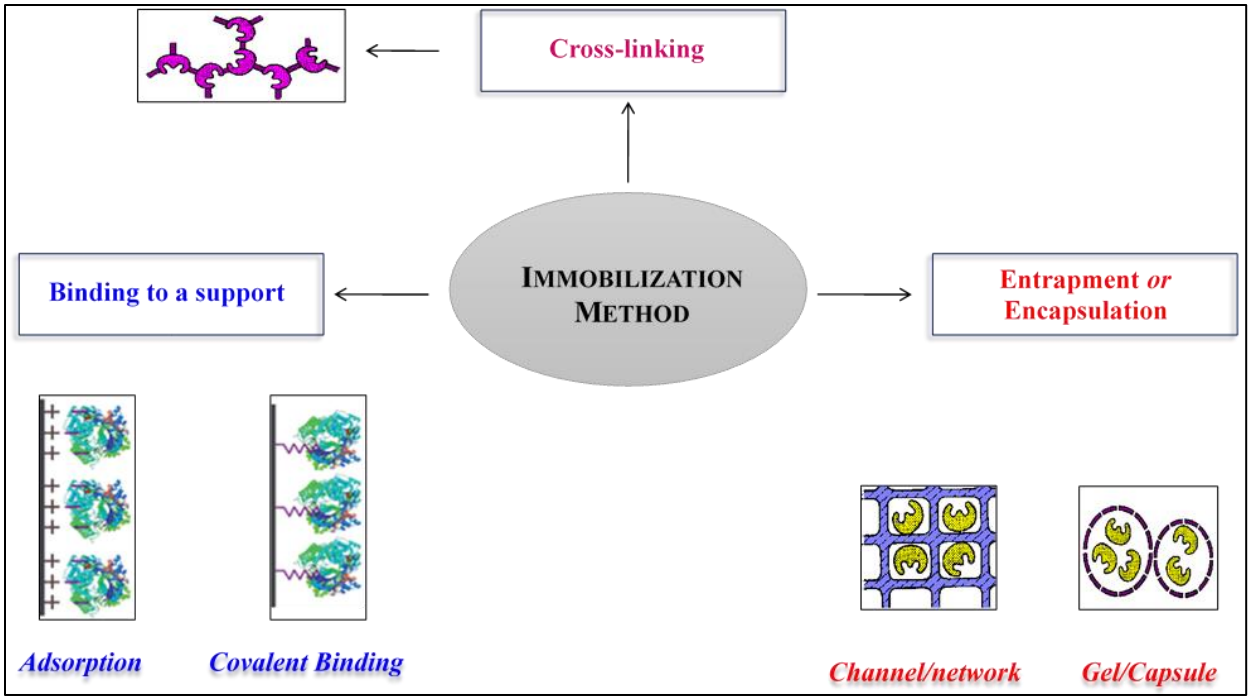


Figure 2.6. Various methods for the immobilization of enzymes (Hwang and Gu, 2012).

2.4.2.4. Self-Immobilization

Self-immobilization or carrier-free immobilization of enzymes avoids limitations that may be associated with the use of carriers or support matrices. Moreover, the use of carriers in immobilization may affect the specific activity or the volumetric activity of the biocatalyst. Self-immobilization is carried out by cross-linking enzymes using a bi-functional cross-linking agent (Brady and Jordaan, 2009). There are various approaches that are used for the self-immobilization of enzymes. Cross-linked enzyme crystals (CLECs) are synthesized by linking protein crystals using a suitable cross-linker; these immobilized enzymes have been reported to show a higher catalytic activity and stability as compared to the free ones. The major limitation of this method is that it requires enzyme crystals which impose the need for highly purified enzyme preparations, resulting in an increase in operational cost (Sheldon, 2007; Brady and Jordaan, 2009).

2.4.2.5. Encapsulation

The encapsulation of enzymes is similar to entrapment and the terms are often used interchangeably. However, encapsulation allows free movement of the enzyme confined in a semi-permeable membrane. This method of immobilization is the most suitable approach to avoid any

detrimental effects on the native structure of the enzymes (Daâssi *et al*., 2014). However, even though this method allows for free diffusion of small biomolecules, it may lead to rupture of the membrane caused by the accumulation of product molecules. The major advantage associated with this method is that it offers a semi-permeable membrane that allows the movement of substrates and products, while limiting leakage of the enzyme from the matrix. The membrane modification in terms of porosity opens wider applicability to various biomolecules to be encapsulated (Bickerstaff, 1997; Guisan, 2006). Alternatively, the mass transfer limitations associated with this matrix could be overcome by use of an alternative approach known as, microencapsulation. Microencapsulation is a technique used to confine a bioactive agent in the core of micron-sized particles made of semi-permeable membranes where, the release of the microencapsulated agents takes place under controlled rates and specific conditions (Desai and Park, 2005; Fernández-Fernández *et al.,* 2013).

2.4.3. Encapsulation of laccase

The encapsulation of laccase has been carried out using various support matrices (Table 2.3) including, organic polymers such as poly-ethyleneimine, alginates, chitin, and gelatin as well as inorganic ones, composed of silica particles. Laccase may be encapsulated in gels (alginates) or in micro or nanoparticles (sol-gels). Recently, laccase has also been reported to be encapsulated by a layer by layer (LbL) technique and in CLECs (Fernández-Fernández *et al.,* 2013). Alginates are the most widely used polymer supports for encapsulation and are usually formed by their cross-linkage with divalent linkers such as calcium chloride, barium chloride etc. Khani *et al.* (2006) reported the encapsulation of laccase and glucose oxidase in alginate carbon beads for applications in biofuel technology, where they indicated that the addition of carbon to the alginate gel enhanced its loading capacity and the half-life of laccase from 75 to 83% and from 38 to 74 days, respectively.

Furthermore, Dominquez *et al*. (2007) reported the effect of selected inducers on the laccase activity after immobilization in alginate beads; the results showed that the highest enzyme activities were achieved by the simultaneous use of the veratryl alcohol and copper sulphate, which were 24-fold higher than the ones obtained without the use of inducers.

Table 2.2. Laccase immobilized using various support matrices (Fernández-Fernández *et al*., 2013).

Laccase source	Support	Substrate	Application	References
Entrapment				
Trametes villosa	Polyaniline matrix	Polycatechol	Biosensor	(Timur *et al*., 2004)
	Cu or Ca alginate beads	4-methoxybenzyl alcohol	Dye decolourization	(Brandi *et al*., 2006)
Trametes versicolor	Polypyrrole matrix	ABTS	Biofuel cells	(Merle *et al*., 2008)
	Sol-gel matrix	Phenols	Biosensor	(Montereali *et al*., 2010)
Adsorption				
Trametes hirsuta	Graphite electrodes	Catechol	Biosensor	(Shleev *et al*., 2006)
Trametes villosa	Aluminium hydroxide		Xenobiotics	(Ahn *et al*., 2007)
Cerrena unicolor	Granocel	ABTS, Syringaldazine		(Rekuc *et al*., 2008)
Trametes versicolor	Magnetic beads		Dye decolourization	(Bayramoglu *et al*.,2010)
Covalent binding				
Trametes villosa	Eupergit C and activated carbon	4-methoxybenzylalcohol		(Brandi et al., 2006)
Trametes versicolor	EDC activated chitosan	ABTS	Xenobiotics	(Cabana *et al*., 2011)
Coriolus versicolor	Chitosan		Xenobiotics	(Zhang *et al*., 2008)
Self-Immobilization				
Trametes versicolor	EPES-CLEAs	ABTS		(Hassani *et al*., 2013)
Myceliophtora thermophila	Spherezymes	ABTS		(Jordaan *et al*., 2009)
Trametes versicolor	CLEAs with PEG or N-oxy	ABTS	Xenobiotics	(Matijosyte *et al*., 2010)
Shewanella putrefaciens	CLEAs	ABTS, Syringaldazine	Dye decolourization	(Sinirlioglu *et al*., 2013)

Lu *et al.* (2007) investigated the effect of alginate-chitosan microcapsules on the activity and stability of laccase, where the results indicated a decrease in the enzymatic activity of the immobilized enzyme, reported with an enhanced temperature and pH stability.

In general, the cross-linkage of alginate with their respective cations is not very strong which could result in the leakage of the catalyst from the encapsulation matrix. Additionally, such linkage is reported to be unstable due to its sensitivity to physiological parameters such as surrounding buffer concentration, thereby resulting in the extraction of the cross-linked cation and subsequent liquification of the alginate (Taquieddin and Amiji, 2004).

The limitations associated with the use of such organic polymers have led to advancement in microencapsulation technologies. Mohidem and Mat (2009) reported the catalytic activity of laccase immobilized in silica sol-gel matrix, where an improved pH stability was obtained for the immobilized enzyme. Furthermore, these authors investigated the effect of additives on the catalytic activity and stability of laccase immobilized in sol-gels; the use of additives resulted in an enhanced catalytic activity (Mohidem and Mat, 2012a). The catalytic activity of laccase in non-conventional solvents was again reported by Mohidem and Mat (2012b) by immobilization in sol-gel laccase (SOLAC), where SOLAC demonstrated a much higher activity and stability in ionic liquids and organic solvents as compared to the free one.

The immobilization of biomolecules in inorganic silica sol-gels (Fig. 2.7) is a fairly established method and has been performed at a large-scale since the early 1990s (Vila-Real *et al*., 2010); the silica sol-gel has been successfully used as an immobilization matrix for various enzymes, including lipase, horseradish peroxidase, alkaline phosphatase, β-glucosidase, chitinase and laccase (Gill and Ballesteros 2000; Qiu and Huang, 2010; Li *et al*., 2011; Mohidem and Mat, 2012a). In comparison to other entrapment matrices, the silica sol-gels offer various advantages such as, inertness and chemical stability, high porosity, optical transparency and ease of preparation. In addition, sol-gel encapsulated enzymes have also been associated with an enhanced enzyme stability and re-usability, thereby making them favourable for industrial applications (Qiu and Huang, 2010).

Table 2.3. Laccase immobilized by encapsulation (Duran *et al*., 2002; Fernández-Fernández *et al*., 2013).

Laccase source	Support	Substrate	Application	References
Trametes versicolor	Alginate-carbon	ABTS	Biofuel cells	(Khani *et al*., 2006)
	Aginate	Reactive dyes	Dye decolourization	(Ramsay *et al*., 2005)
	Sol-gel	2,6-dichlorophenol		(Mohidem and Mat, 2009)
	Poly(ethyleimine)			(Zhang and Rochefort, 2010)
	Alumina pellets	ABTS	Paper industry	(Crestini *et al*., 2010)
	Poly(ethyleimine)	Paper substrates	Biosensors	(Zhang and Rochefort, 2011)
	Sol-gel	2,6-DMP	Phenolic pollutants	(Mohidem and Mat, 2012ab;
		Ferulic acid		Gill *et al*., 2018a,b)
Coriolus versicolor	Calcium alginate	Chlorophenols	Xenobiotics	(Zhang *et al*., 2006)
Polyborus rubidus	Na-alginate	Synthetic dyes	Dye treatment	(Dayaram and Dasgupta, 2008)
Myceliapthora thermophile	Alginate/gelatin	Phenols	Dye decolourization	(Wang *et al*., 2008)

The preparation of silica sol-gels (Fig. 2.7) occurs via three main steps: preparation of the silica sol, gelation and aging. The silica sol is formed by the acid or base-catalyzed hydrolysis of a silica precursor, which involves the condensation and poly-condensation of silanols to produce siloxane bonds as well as water and alcohol as *by*-products. In order to allow gelation of the silica sol, an increase in the pH is required which is carried out by the addition of a buffer, following which, the enzyme to be encapsulated is introduced. The gel is then aged to promote strengthening of the silica gel network and later dried for solvent removal, leading to the formation of a xerogel (Vila-Real *et al*., 2010). Despite the several advantages offered by this entrapment matrix, the silica sol-gels are often associated with certain limitations, including volume shrinkage, brittleness and cracking, excessive alcohol production and enhanced mass-transfer limitations (Vila-Real *et al*., 2010; Mansor *et al*., 2016).

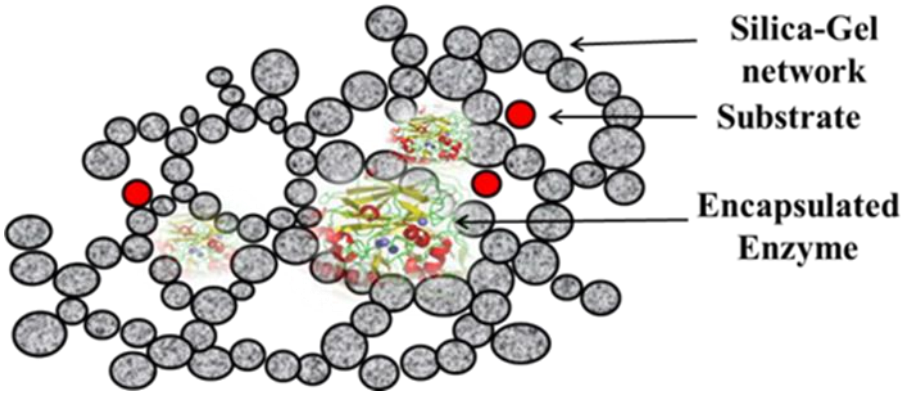


Figure 2.7. Encapsulation of enzymes in silica sol-gel matrix (Pierre, 2004).

2.4.4. Optimization of encapsulation variables using response surface methodology

Response surface methodology (RSM) is a combination of statistical and mathematical equations that is useful for developing, improving and optimizing processes (Fig. 2.8). RSM studies are applied for designing, formulation and improvement of new and existing products (Aslan, 2008). This methodology has several advantages over classic experimental techniques that make use of one variable at a time and does not require a large number of experiments for optimization, while providing a large amount of information (Bezera *et al*., 2008).

RSM makes use of two important designs, namely central composite design (CCD) and Box-Behnken design (BBD). The CCD is considered as the most ideal design for sequential

experimentation and provides almost the same amount of information as a three-level factorial, however requiring fewer experiments than the full factorial design (Aslan, 2008; Demirel and Kayan, 2012). Generally, various parameters may affect the encapsulation process and hence, screening experiments could be carried out to identify the parameters that have the most significant effects on the process (Paulo and Santos, 2017).

Lu *et al*. (2009) investigated the optimization of selected parameters for the silica-sol gel immobilization of trypsin using a CCD and indicated a residual enzyme activity of 35.6% as well as encapsulation efficiency (EE) of 68.3%, under optimized conditions. Lettera *et al*. (2016) reported the optimization of the covalent immobilization of laccase, from *Pleurotus ostreatus*, using BBD; these authors reported a well fitted process by a quadratic polynomial equation, with a coefficient of determination (R^2) of 0.94 and 98% immobilization efficiency. Similarly, Mansor *et al*. (2016) optimized the sol-gel microparticles synthesis conditions for the encapsulation of laccase, obtained from *Trametes versicolor*, using the BBD; the results showed that the optimal conditions were obtained with a water to silane ratio of 5.44, 2.52 µmol hydrochloric acid (HCl) concentration, 0.39 mmol tetraethylamine and 3.83 mg/mL enzyme loading. These authors (Mansor *et al*., 2016) also indicated the predicted and observed responses (residual laccase activity) of 301.7 and 298.36 U/g with a R^2 of 0.89, which may demonstrate a moderate degree of correlation between the response and independent synthesis variables.

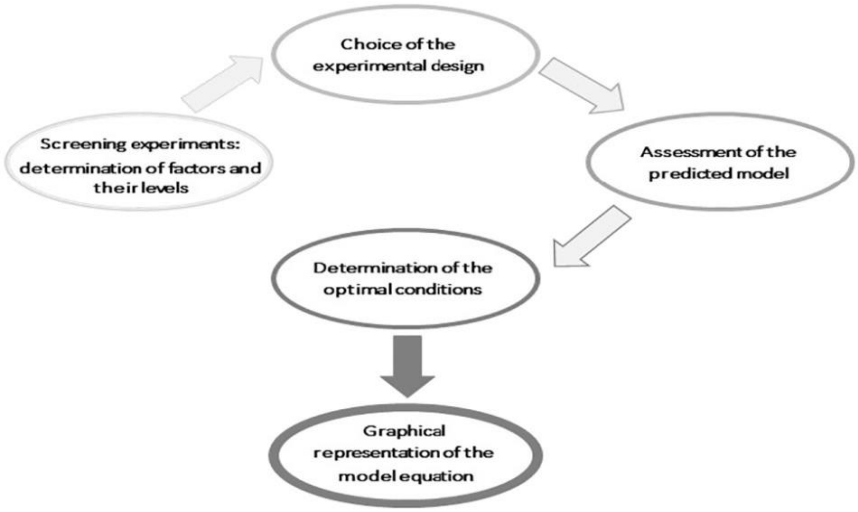


Figure 2.8. The process of optimization using response surface methodology (RSM) (Paulo and Santos, 2017).

2.5. Biocatalysis in non-conventional media

2.5.1. Generality

Aqueous media are the traditional solvent systems for enzyme-catalyzed transformations; however, the use of aqueous media limits their applications for large scale industrial processes. Contrarily, organic solvent systems offer several advantages as compared to aqueous ones: (i) higher solubility for several organic compounds; (ii) greater stability of biocatalysts; (iii) ease of product recovery; (iv) facilitating the reuse of enzymes due to their insolubility in organic media and (v) allowing for cost efficient industrial processes (Zaks and Klibanov, 1985). However, these properties are not applicable for all biocatalysts and organic solvent systems may sometimes denature the enzymes leading to mass-transfer limitations, resulting from complexity in reactions (Adlercreutz, 2000; Doukyu and Ogino, 2010).

In addition, organic solvents are also associated with hazardous activities which are a concern for the environment and hence there is a pressing need for the development of greener and sustainable technologies for efficient bioconversions (Sheldon, 2005). The utilization of 'Green chemistry' reduces or eliminates the use of such chemicals for manufacturing purposes. There have been five major green solvent systems described in the literature: (i) supercritical fluids, (ii) fluorinated solvents, (iii) ionic liquids, (iv) water and (v) solvent-free systems (Hobbs and Thomas, 2007; Ghaffari-Moghaddam *et al*., 2015).

2.5.2. Parameters affecting enzymatic catalysis in non-conventional media

2.5.2.1. Effect of water activity (a_w)

A fundamental factor that could affect enzymes in non-conventional solvent systems is the optimal level of hydration in the surrounding solvent medium. Water is involved in all non-covalent interactions that maintain the active enzymatic conformation and hence is of utmost importance for biocatalytic reactions. In addition, the complete dehydration of enzymes is impossible as there will be residual water that is tightly bound to the enzyme and limits its mobility in terms of forming hydrogen bonds in hydrophobic environments (Gorman and Dordick, 1991). Zaks and Klibanov (1988) investigated the effect of organic solvents on the activity of enzymes and indicated that the enzyme catalytic activity was only a function of the amount of bound water and not to that present in the surrounding solvent medium.

The a_w may be measured by the vapor phase above the reaction mixture and is expressed as the partial pressure of the solution over that of pure water (Valivety *et al.*, 1992). In addition, the use of salt pair hydrates can be used to control the a_w and is based on the fact, that a hydrated salt and its corresponding lower hydrate are inter-converted at a fixed a_w while maintaining near optimal a_w control in a change of solvents, reactants or enzyme concentration (Kvittingen *et al.*, 1992).

2.5.2.2. Effect of pH

The pH of the surrounding medium has comparatively less significance in non-conventional solvent biocatalysis. It is observed that enzymes demonstrate a 'pH memory' in organic solvents, where the enzymatic catalytic activity reflects the pH of the last aqueous solution in which they were exposed. This property could be due to the ionized group of enzymes that retain its last ionization state after dehydration and a subsequent exposure to an organic solvent. Alternatively, in systems of minimal water content, the addition of an appropriate buffering system could enhance the enzyme's catalytic activity (Klibanov, 2001).

2.5.2.3. Effect of reaction temperature

Temperature affects most biochemical reactions and usually an increase in temperature could result in an enhancement of the reaction rate; however, the use of relatively higher temperatures can also reduce the reaction rates by leading to the denaturation of the enzymes (Stamatis *et al.*, 2001). The temperature is strongly affected by the enzyme's stability requirements, where the optimum temperature must compromise with other factors such as the economic costs, processing conditions and productivity. Even though higher temperatures enhance the reaction rate, the substrate-product diffusion and the substrate viscosity, mild reaction conditions are preferable for most bioprocesses (Serrano-Arnaldos *et al*., 2016).

2.5.2.4. Effect of substrate concentration

In general, different solvents show different abilities to solubilize substrates, which further influences the enzyme's catalytic activity (Wehtje and Adlercreutz, 1997). In case of poorly active enzymes, the reaction kinetics are usually limited by their corresponding catalytic activity; however, this may not be the case for relatively highly active enzymes, where such reaction kinetics could be limited by substrate diffusion (Illanes, 2008). Such diffusional limitations could

be overcome using increased agitation speeds, temperature and interfacial surface areas (Dordick, 1989; Klibanov, 2001; Illanes, 2008).

2.5.2.5. Effect of agitation speed

The proper agitation of the reaction components at an optimum speed plays a fundamental role in the enhancement of mass-transfer in non-conventional solvent systems (Sun *et al*., 2012; Gawas *et al*., 2016). The use of agitation in biocatalysis results in an increase in the mixing of reactants that could be due to a simultaneous increase in the collision between their particles (Yadav and Thorat, 2012).

Yadav and Thorat (2012) investigated the effect of external mass-transfer of the lipase catalyzed synthesis of isoamyl myristate from isoamyl alcohol and myristic acid, in SFM, within a range of selected agitation speed; the results showed that the initial reaction rate increased with a simultaneous increase in the agitation speed up to a certain point, beyond which there was a marginal decrease which may be due to the damage caused to the immobilized biocatalyst (Yadav and Thorat, 2012).

2.5.3. Biocatalysis in solvent-free media (SFM)

Solvents provide several advantages for industrial processes for the transportation of solids, dissolving immiscible regents, reducing the viscosity of the reaction medium and overcoming mass transfer-limitations. In addition, solvents also aid in the dissipation of the reaction heat and promote enhanced selectivity, when used as diluents. However, organic solvents are a major source of volatile organic compounds and could lead to relatively higher costs in enzyme-catalyzed processes (Yadav and Thorat, 2012). In order to overcome such limitations, the use of solvent-free media is preferred, where an absence of solvents facilitates downstream processing, is environmentally friendly and cost effective (Yadav and Thorat, 2012). Moreover, the use of solvent-free media has been associated with increased volumetric productions that could make them an attractive choice for industrial applications (Santos *et al*., 2007).

Solvent-free reactions may be defined as those that use less than 5 equivalents of one solvent or reagent, with respect to the substrate (Walsh *et al*., 2007). The major drawback of reactions carried out in SFM is the higher viscosity of reaction components that may ultimately cause mass-transfer limitations (Sandoval *et al.*, 2002). Generally, the SFM may be of two types; (i) one of the reagents

is a liquid or in gaseous form, for gases and are known as heterogenous eutectic reactions, (ii) the reagents are in solid form and are known as solid to solid biocatalysis. The solid state biocatalysis has potential advantages in comparison to the heterogenous reactions, where they require smaller reactor volumes and increased cost savings. However, solid state biocatalysis are often characterized by mass transfer limitations with lower reaction rates and would require chromatographic means for the separation of the products and reactants (Hobbs and Thomas, 2007).

Several studies have been reported for the transesterification reactions using immobilized lipases in SFM, which make use of liquid substrates such as oils (Chaibakhsh *et al*., 2009; Aziz *et al.*, 2012; Sorour *et al*., 2012a,b; Sun *et al*., 2012; Gawas *et al*., 2016). Even though these reactions may be similar to those carried out in conventional aqueous or organic solvents, they showed relatively higher bioconversions and initial reaction rates due to an increased viscosity of the mediums. However, there is no literature for the biosynthesis of oligo or polyphenols that are carried out in SFM.

Chaibhakhsh *et al.* (2009) reported the optimization of lipase-catalyzed synthesis of adipate ester in SFM, where they indicated an increased volumetric productivity and bioconversion yield as compared to that in the organic solvent medium. Likewise, Sorour *et al*. (2012a) investigated the lipase-catalyzed synthesis of phenolic lipids in SFM and indicated an enhanced bioconversion yield for the transesterification of flaxseed oil as well as a significantly increased volumetric productivity.

Although the use of SFM could avoid detrimental effects on the biocatalyst, as compared to the reactions carried out in organic solvents, there may be other implications; the surrounding media would change as the reaction proceeds, where the synthesized end-products would be the new reaction media for the biocatalyst. In such cases, the impact of the reactions is unpredictable and complicated hence indicating the importance of the development of a suitable biocatalyst by immobilization (Walsh *et al*., 2007).

2.6. Structure elucidation and characterization of phenolic compounds

2.6.1. Chromatographic techniques

There are several chromatographic techniques used for the efficient purification and separation of phenolic compounds, mainly including size-exclusion chromatography (SEC) and high-performance liquid chromatography (HPLC).

2.6.1.1. Size-exclusion chromatography (SEC)

SEC is also known as gel/filtration chromatography and is used for the separation and identification of compounds varying in their molecular weight and sizes. The use of SEC for the separation of the end products of the laccase-catalyzed transformation of FA was investigated by Bou-Mitri (2013); the author reported the use of Trisacryle GF05 M gel size-exclusion column (2.4 x 90 cm) for the purification of the end-products obtained in both aqueous and organic solvent medium. The separation of the end products of the lignin peroxidase-catalyzed transformation of FA was also reported by Ward *et al*. (2001) using TSK gel® G3000 HR column, where the elution was performed using tetrahydrofuran (THF) as the mobile phase with a flow rate of 0.5 mL/min. Kermasha *et al.* (2002) and Hossain (2004) reported a Trisacryle GF05 gel size exclusion column procedure for the purification of the tyrosinase-catalyzed oxidative products of catechin.

2.6.1.2. High-performance liquid chromatography (HPLC)

HPLC is the most preferred method for the quantification and identification of polyphenol extracts, where the principle chromatographic conditions used are a reverse-phase C18 column, UV-Vis diode array detector and a binary solvent system consisting of an acidified solvent (Solvent A) and a polar organic solvent (Solvent B). Osman *et al*. (2007) used HPLC with a reverse phase column for the separation and identification of laccase-catalyzed end products from catechin. Furthermore, such study was also carried out using other substrates such as FA (Carunchio *et al*., 2001) and vanillic acid (Bollag *et al*., 1982). Adelakun *et al*. (2012a) carried out the HPLC separation of the products obtained in laccase-catalyzed oxidation of FA in monophasic and biphasic solvent systems, using a reversed phase LUNA 5µ PFP (2) column under isocratic conditions of acetonitrile:water:acetic acid (25:75:0.1, v/v/v) as the mobile phase. Likewise, Bou-Mitri (2013) characterized the molecular weights of the laccase-catalyzed end-products from FA, synthesized

in aqueous and organic solvent media that were analyzed on a polydivinilbenzene column, using a mobile phase of 100% THF.

2.6.2. Spectral methods

2.6.2.1. Fourier transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is a powerful technique which identifies chemical bonds and functional groups in a molecule, while producing a "fingerprint" of the same. FTIR is based on the difference in the vibration frequencies of given specific bonds and hence, the wavelength of the light absorbed is characteristic of the particular chemical bond in the spectra obtained (Griffiths and Hasseth, 2007).

Ma *et al.* (2009) investigated the *Rhus vernificera* laccase-catalyzed oxidation of phenolic compounds, i.e., catechin, epicatechin and catechol in organic solvents; FTIR was used for the structural characterization of the catalyzed end-products and the results indicated the formation of a probable dimer from catechin in the organic solvent medium. Tan and Shahidi (2011) reported a synthesis of two phytosteryl ferulates, which were confirmed using FTIR for their characterization. Bou-Mitri (2013) conducted the FTIR analysis of the purified end-products of the laccase-catalyzed oxidation of FA using a Bruker Alpha-P spectrometer at 4 cm^{-1} resolution; the results indicated that the reaction medium had a modulating effect on the regio-selectivity of the end-products and involved different coupling modes for the free phenoxy radicals for the aqueous and organic solvent medium.

2.6.2.2. Mass spectrometry (MS)

Mass spectrometry is used for elucidation of chemical structures of various molecules such as peptide, polyphenols etc. The basic principle of MS relies on ionization of chemical compounds for generating charged ions and subsequently measuring their mass to charge ratio (*m/z*) (Ignat *et al*., 2011). It is also coupled to chromatography (such as LC-MS and GC-MS) for more effective analysis of the structures involved. GC-MS is usually coupled with pyrolysis and involves the thermal fragmentation of the compound of interest in the absence of molecular oxygen; these volatile molecules are then analyzed by Pyrolysis/MS after separation in GC/MS columns (Bou-Mitri, 2013). The technique requires the use of relatively smaller samples sizes (10-1000 mg) and is rapid as compared to other methods (Hossain, 2004). MS was used for the characterization of

oxidation reaction involving pentachlorophenol and FA by lignin degrading enzymes (Ruttiman-Johnson and Lamar, 1996). Ward *et al.* (2001) used the GC/MS for the structural characterization of FA polymerization by lignin peroxidase. Kermasha *et al.* (2002) and Hossain (2004) reported Pyrolysis/GC/MS analysis of polyphenol oxidase catalyzed end products from phenolic extracts in selected organic and aqueous solvent systems.

2.6.3. Determination of antioxidant capacity of phenolic compounds

The antioxidant capacity is the ability of certain compounds to protect a biological system against potentially harmful effects of oxidation reactions that involve free radical oxygen and nitrogen species (ROS and RNS). The various methods to measure the antioxidant capacity may differ in terms of the reaction mechanisms, the oxidant and target/probe species and the reaction conditions (Karadag *et al*., 2009). The methods used to measure this capacity are, the Trolox equivalent antioxidant capacity (TEAC), total radical absorption potentials (TRAP), ferric reducing/antioxidant power (FRAP), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and oxygen radical absorption capacity (ORAC) assays (Prior *et al*., 2005; Karadag *et al*., 2009).

ORAC is one of the most widely used antioxidant assays, where its principle relies on the measurement of inhibition of peroxyl radicals that are generated from thermal decomposition of 2,2'-Azobis (2-amidinopropane) dihydrochloride (AAPH azo-initiator) in aqueous buffer or hydroxyl radicals which are generated by the cupric ion-H_2O_2. Furthermore, the peroxyl radicals react with a fluorescent probe forming a non-fluorescent product, resulting from the quenching of the fluorescent molecules. The antioxidant capacity of the compound is determined by the delayed quenching of the probe, which is determined by the radical scavenging capacity of the investigated compound (Karadag *et al*., 2009). Trolox, a vitamin E analogue of five different concentrations is used as a standard to prepare a standard curve by incubating it with fluorescein at 37°C before the addition of AAPH and the decay of fluorescence is measured after every minute for a period of 35 minutes resulting from the reaction with peroxyl radicals. The data is expressed as micro moles of TE (trolox equivalents)/L or /g of the sample (µmol of TE/g or µmol of TE/L) (Prior *et al.,* 2005; Karadag *et al.,* 2009). The ORAC assay consists of model reactions that mimic both *in vivo* and *in vitro* systems (Aziz, 2013; Bou-Mitri, 2013). The antioxidant capacity of a compound could also be measured by determining the reducing ability of the compound towards DPPH, where its free radical is an organic nitrogen radical bearing a deep purple colour. The free radical scavenging

capacity is measured by electron spin resonance or by following the absorbance decrease at 515 nm and the percentage of DPPH remaining is considered directly proportional to the antioxidant concentration. The advantage of using the DPPH assay is its simplicity and reduced time for the analysis of the antioxidant capacity. However, there are certain limitations associated with this method; (i) The analysis of hydrophilic compounds is not possible as DPPH is only soluble in organic solvents, (ii) The method does not represent the antioxidant capacity *in vivo*, since DPPH is not similar to peroxyl radicals, (iii) In addition, compounds such carotenoids may demonstrate an overlapping absorbance at 515 nm which could cause complications in the interpretation (Prior *et al*., 2005; Karadag *et al*., 2009).

Tan and Shahidi (2013) carried out chemoenzymatic synthesis of novel phytosteryl phenolates, where the antioxidant capacity for both compounds was investigated using the ORAC; the results showed higher antioxidant capacities of the synthesized products as compared to intial compounds, vanillic acid and vinyl vanillate. The oligomerization of FA reported by Bou-Mitri (2013) in an organic monophasic system exhibited the synthesis of two dimers; the antioxidant capacity of the dimers was investigated using ORAC and the results indicated a higher antioxidant capacity of phenolic oligomers as compared to that for the FA.

CONNECTING STATEMENT TO CHAPTER III

Chapter III covers the research work aimed at the encapsulation of the laccase enzymatic extract, obtained from *Coriolus hirsutus*, in selected matrices, including alginate, alginate-silica and silica sol-gel. For the assessment of the matrices, their encapsulation efficiency and residual laccase activity were determined. In addition, the encapsulated laccase was investigated for its kinetic parameters, protein leakage, re-usability and storage stability.

CHAPTER III

OPTIMIZATION OF ENCAPSULATION OF A MICROBIAL LACCASE ENZYMATIC EXTRACT USING SELECTED MATRICES

3.1. Abstract

The encapsulation of the laccase extract, obtained from *Coriolus hirsutus*, was investigated using selected matrices, including alginate, alginate-silica and silica sol-gel. Two methods, M1 and M2, were developed for the hybridization of alginate with silica. The experimental findings indicated that the incorporation of silica into alginate, using M2, resulted in a higher (70%) encapsulation efficiency (EE) for the laccase extract as compared to that for the alginate (59%) alone. Furthermore, the encapsulation of the laccase extract in sol-gel resulted in an enhancement of its catalytic activity, with a concomitant increase (90%) in the EE. The alginate and sol-gel matrices also enhanced the laccase catalytic efficiency as compared to that for the free one, with a k_{cat} of 89.9, 63.7 and 56.9 min^{-1}, respectively. The protein release profiles indicated that the laccase was more effectively confined in the sol-gel and alginate-silica M2 as compared to that for the alginate and alginate-silica M1. In addition, the laccase extract in sol-gel exhibited an enhanced re-usability, with a relative residual enzyme activity of 82.7%, after 5 continuous reaction cycles. The alginate, alginate-silica M2 and sol-gel matrices demonstrated complete storage stability at 4°C, without any loss in laccase activity after 6 weeks of storage.

3.2. Introduction

Laccases (EC 1.10.3.2) are multi-copper proteins that catalyze the oxidation of various aromatic and non-aromatic compounds, using molecular oxygen as a co-factor (Jaiswal *et al*., 2014). The occurrence of laccases in nature is abundant, where they have been found in several species of fungi, bacteria and higher plants; laccases are mainly associated with delignification, pigment formation and detoxification (Strong and Claus, 2011). Even though laccases show a wide range of substrate specificity, their use in various industrial applications was often hindered by their high production costs and poor stability, as well as difficulties in their recovery and re-usability (Yamak *et al*., 2009; Strong and Claus, 2011). Nevertheless, such limitations could be overcome by the use of suitable methods of immobilization, which are aimed at a more efficient and economical use of the enzymes (Zhang *et al*., 2013). The immobilization of laccase has been reported extensively,

using different methods and supports (Fernández-Fernández *et al*., 2013; Guzik *et al*., 2014), where the encapsulation of enzymes in a semi-permeable matrix may be considered as one of the most appropriate approaches, as it offers relatively mild process conditions and little or no effect to their native structure (Daâssi *et al*., 2014).

Among the various matrices used for the encapsulation of laccase, alginate is one of the most commonly employed biopolymers, owing to its mild gelling properties and non-toxicity; however, the encapsulation in alginate gels is mostly associated with a lower stability and an uncontrolled porosity (Coradin *et al*., 2003). In order to develop more robust and stable matrices, the incorporation of silica into alginate has been investigated for the encapsulation of biomolecules (Coradin *et al*., 2003; Meunier *et al*., 2010).

Recently, Mohidem and Mat (2012a) reported that the encapsulation of laccase in inorganic silica gels has been linked to an enhancement of its catalytic efficiency. In addition, the silica sol-gel matrices offer several other advantages, including non-toxicity, improved enzyme stability, optical transparency, enhanced re-usability and storage stability, as well as the possibility of tuning the gel porosity (Vila-Real *et al*., 2010; Lloret *et al*., 2011).

Previous studies (Jaiswal *et al*., 2014; Daâssi *et al*., 2014; Mohidem and Mat, 2012ab; Lloret *et al*., 2011, Xu *et al*., 2006b, Asgher *et al*., 2012) have mostly reported the use of the commercial laccase extract either from microbial sources other than *Coriolus hirsutus*, including, T*rametes* species, *Coriolopsis gallica*, *Leucaena leucocephala*, *Pleurotus ostreatus*, *Miceliophthora thermophilca* or from other unknown microbial sources. Moreover, the laccase used throughout this study was an ultra-filtrate enzyme extract, obtained without the use of any diluent as that reported by Mohidem and Mat (2012b).

The overall objective of this study was to investigate the encapsulation of an enzymatic laccase extract, from *Coriolus hirsutus,* in selected matrices, including alginate, alginate-silica and silica sol-gel. The matrices were assessed in terms of their encapsulation efficiency and residual laccase activity. In addition, the kinetic parameters, protein leakage, re-usability and storage stability of the entrapped laccase were also investigated.

3.3. Materials and Methods

3.3.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). Ferulic acid (FA), bovine serum albumin (BSA), tetraethyl orthosilicate (TEOS) (98% purity) and calcium chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). Sodium alginate and all organic reagents of HPLC grade or higher were obtained from Fischer Scientific (Fair Lawn, N. J.). Syringes and needles were purchased from BD Syringes and Needles (Mississauga, ON). All reagents and buffers were prepared in deionized water, using Milli Q plus (Millipore).

3.3.2. Production of Coriolus hirsutus biomass

Coriolus hirsutus was maintained on malt agar media plates and incubated at 20°C as per the ATCC protocol. The production of the *Coriolus hirsutus* biomass was carried out according to the procedure described by Taqi (2012). The fermentation was initiated by the inoculation of the *C. hirsutus* on malt agar and used for the pre-culture. The pre-culture was incubated at 28°C for 7 days, at 160 rpm following which; it was poured into fresh media and incubated under the same conditions for a period of 6 days.

3.3.3. Recovery and enrichment of the laccase enzymatic extract

The recovery and enrichment of the enzyme was performed as per Taqi (2012). Briefly, the mycelium pellets were filtered, and laccase was recovered by ultra-filtration using a Prep/Scale TFF cartridge (Millipore) with polyethersulfone membrane of 10 *k*Da cut-off filter and a pressure of 10 psi. All protocols were performed at 4°C and the enriched enzymatic extract was lyophilized in the presence of mannitol (2.5% w/v) and stored at -80°C for further analysis.

3.3.4. Encapsulation of the laccase enzymatic extract in selected matrices

3.3.4.1. Encapsulation of the laccase extract in alginate matrix

The encapsulation of laccase extract in alginate was carried out according to the procedure described by Bou-Mitri (2013), with certain modifications. The enzymatic laccase suspension (1 mg/mL protein) was prepared in glycine-sodium hydroxide buffer (0.2 M, pH 9.5) and mixed with

an alginate solution of 1.5% (w/v), to a final protein to alginate ratio of 1:15 (w/w). The mixture was dropped into the calcium chloride solution (0.1 M) following which, the calcium alginate beads formed were hardened for 1 h at 4°C, under continuous stirring. Subsequently, the encapsulated laccase was recovered by suction filtration on Whatman filter paper (No. 4) and washed with deionized water. The beads were then washed with acetone, dried using a gentle stream of nitrogen and stored at -20°C for further assays. The filtered calcium chloride and the washing solutions were collected for protein determination.

3.3.4.2. Encapsulation of the laccase extract in alginate-silica matrix

Two methods were used to encapsulate laccase in alginate-silica matrix. The first method (M1) was carried out as described by Heichai-Segal *et al.* (1995). The calcium alginate beads containing laccase were prepared as described previously; however, the beads were recovered after 5 minutes and immediately added to a solution of hexane and the silica precursor (TEOS) (v/v). The incubation was carried out overnight at 4°C, to allow the diffusion of silica precursor into the alginate gel, forming alginate-silica hybrids. The alginate-silica beads were then filtered and washed thoroughly to remove any unreacted silica.

The second method (M2) was performed as per the protocol outlined by Xu *et al.* (2007), with certain modifications. The method involved the incubation of calcium alginate beads in a pre-hydrolyzed silica sol, which was prepared by the ultrasonication and vigorous stirring of TEOS (11 mmol), deionised water (22 mmol) and 0.04 M hydrochloric acid (HCl) (0.004 mmol), until the formation of a clear solution. The prepared alginate beads were recovered as described for M1 following which, they were added to the pre-hydrolyzed sol for 15 minutes. The cured beads were later recovered, washed and re-suspended in the calcium chloride solution for further hardening. All supernatants and washing solutions were collected for protein determination.

3.3.4.3. Encapsulation of the laccase extract in silica sol-gel matrix

The silica sol-gel encapsulation of the laccase extract was carried out as described by Mohidem and Mat (2012a,b) with modifications. A pre-hydrolyzed sol of TEOS, deionised water and HCl was prepared, as described previously. Laccase extract (1 mg protein/mL of silica sol) was suspended in potassium phosphate buffer (0.01 M, pH 6.0) and added to the silica sol. In addition, this was followed by an immediate addition of the potassium phosphate buffer (0.4 M, pH 7.0) to

facilitate gelation and was continuously agitated, until the onset of gelation (1-3 min). The resulting gel was then washed, recovered and lyophilized at -42°C for 6 h and stored at -20°C to maintain its catalytic activity. All washing solutions were collected to analyze their protein content.

3.3.5. Protein determination

The protein content of the free enzyme extract and washing solutions was determined according to a modification to the Lowry method (Hartree, 1972), using bovine serum albumin (BSA) as a standard for the calibration curve.

3.3.6. Enzyme activity of the encapsulated laccase extract

The enzyme activity assay for both free and encapsulated laccase was carried out according to Bou-Mitri (2013). A sample of free or encapsulated laccase extract was suspended in sodium acetate buffer (0.1 M, pH 5.0), and the enzyme activity was measured following the oxidation of FA (30 mM) as a substrate. The decrease in the absorbance at 320 nm was measured spectrophotometrically (Beckman Instruments Inc., San Raman, CA). The specific activity was defined as µmol of converted ferulic acid/mg protein/min at selected time intervals (0 to 10 min). The enzymatic bioconversion was carried out at 50°C under continuous stirring at 150 rpm. All laccase assays were performed in triplicate trials and were run simultaneously with blank reaction containing everything except the enzyme.

The Michaelis-Menten parameters, K_m and V_{max} of the free and encapsulated laccase extract were determined from the Lineweaver-Burk plots and the catalytic efficiency, k_{cat} was calculated from the ratio of V_{max}/K_m. Plots were constructed by measuring laccase activity within a range of FA concentrations (0 to 50 mM). All activity assays were conducted by means of triplicate trials, under the standard conditions, described earlier.

3.3.7. Characterization of the encapsulated laccase enzymatic extract

3.3.7.1. Encapsulation efficiency (EE) and relative (%) of residual laccase activity

The encapsulation efficiency (EE) for the alginate and alginate-silica beads was quantified by the difference between the total initial added protein to the pre-gel solution, and the sum of total released protein into the calcium chloride bath and the washing solutions. In contrast, the EE for the sol-gel matrix was defined as the difference between the total added initial protein to the silica

sol and the determined protein in the recovered washing solutions, respectively. The encapsulation efficiency (%) and the residual enzyme activity (%) were calculated as follows:

$$\text{Encapsulation efficiency (EE) (\%)} = (C_0 V_0 - Cf\, Vf) / (C_0 V_0) \times (100) \quad \text{(I)}$$

$$\text{Residual enzyme activity (\%)} = (SA_{imm}) / (SA_{free}) \times (100) \quad \text{(II)}$$

Where, C_0 and C_f represent the initial loaded protein and the final protein concentrations recovered in the washing solutions, while V_0 and V_f were the initial and final volumes of the enzyme extract suspensions and washing solutions, respectively. SA_{free} and SA_{imm} were the specific activity of the free and encapsulated laccase extract, respectively, and it is defined as µmol substrate (FA)/mg protein/min.

3.3.7.2. Protein leakage of the encapsulated laccase extract

Protein leakage study for the selected matrices was performed for 24 h, according to the method described by Khani *et al.* (2006). Two g of each matrix containing the encapsulated laccase extract, was suspended in 10 mL of sodium acetate buffer solution (0.1 M, pH 5.0) at room temperature and samples were collected at selected intervals, of 1 h for the first 6 h and subsequently, at intervals of 6 h for the remaining storage time.

3.3.7.3. Storage stability of the encapsulated laccase extract

The storage stability of the free and encapsulated laccase extract was investigated at 4°C, for a period of six weeks. The relative residual laccase activity was expressed as its relative percentage at a certain storage time, as compared to that for the initial.

3.3.7.4. Re-usability of the encapsulated laccase extract

In order to investigate the re-usability of the encapsulated laccase, the enzyme extract was re-used for six continuous reaction cycles, under the standard conditions described previously. After each cycle, the encapsulated enzyme extract was washed, recovered and added to a fresh buffer solution containing FA. The relative residual laccase activity of the encapsulated laccase extract was expressed, as the relative percentage laccase activity in a defined cycle, as compared to that for its initial activity.

3.3.8. Statistical analysis

All data were expressed by means of at least three independent trials with their respective standard deviations (SD). The relative standard deviation (RSD) was defined as the SD divided by the mean multiplied by 100. Correlation analyses were performed using Microsoft-Excel (Microsoft) and the data was analyzed by PROC ANOVA analysis, with a post-hoc analysis using Tukey's honest significance test. Values were considered significant, when $P \leq 0.05$.

3.4. Results and Discussion

3.4.1. Encapsulation of the laccase extract in alginate matrix

Table 3.1 shows the summary of the experimental data for the encapsulation of the laccase extract in selected matrices. The encapsulation of the enzyme extract in the alginate matrix resulted in a 59.8% encapsulation efficiency (EE), which was significantly ($P \leq 0.05$) lower than that obtained with the sol-gel (90.4%) and with the alginate-silica matrix M2 (70.7%). In contrast, the residual laccase activity, recovered in the alginate matrix, was relatively higher (80.7%) than that obtained with the alginate-silica hybrids M1 (37.4%) and M2 (68.6%).

Although the experimental findings (Table 3.1) are in agreement with those reported in literature, the residual laccase activity obtained for the alginate entrapped enzyme extract was relatively higher. The difference in the enzyme activity could be due to the glycine-sodium hydroxide buffer, which was used to facilitate the introduction of the laccase extract into the pre-gel alginate solution, by maintaining its pH. In general, the catalytic activity of enzymes is significantly affected by the pH of their surrounding environment; hence the pH value of the gel must preferably be around the optimum pH of the encapsulated enzyme (Betancor and Luckarift, 2008). Bou-Mitri (2013) reported that the optimum pH for laccase extract, from *C. hirsutus*, was 5.0; hence, the initial pH of the pre-gel solution containing the enzyme was therefore adjusted to 5.5. In addition, a pH value less than 5.5 resulted in a viscous pre-gel solution with air bubbles, which could ultimately affect the matrix EE; in contrast, at pH values above 5.5, a relatively low viscosity alginate solution was obtained, which made its handling extremely difficult. Overall, the experimental findings (Table 3.1) suggest that the higher laccase activity could be a result of the use of a more appropriate pH value for the alginate encapsulation of the enzyme extract as compared to that reported in literature (Phetsom *et al*., 2009; Bou-Mitri, 2013).

Table 3.1. Effect of the matrix type on encapsulation of the laccase extract, from *Coriolus hirsutus*, assayed with ferulic acid at, 50°C and pH 5.0.

Encapsulation[a] matrix	Loaded protein (mg)	Encapsulation efficiency (%)[b]	Specific activity[c]	Total activity[d]	Residual laccase activity (%)[e]
Free	2.50 (1.52)[f]	-	16.26 (0.68)	40.78 (1.82)	-
Alginate	1.48 (2.40)	59.80 (2.40)	13.04 (1.79)	19.24 (4.18)	80.74 (1.79)
Alg-Si M1	1.32 (5.34)	53.54 (5.34)	6.04 (0.66)	8.00 (4.67)	37.40 (0.66)
Alg-Si M2	1.75 (2.02)	70.71 (2.02)	11.08 (0.50)	19.39 (2.52)	68.60 (0.50)
Silica sol-gel	2.25 (0.63)	90.40 (0.63)	51.67 (10.75)	116.30 (11.38)	319.92 (10.75)

[a]Encapsulation matrix was the surrounding matrix type for the entrapment of laccase enzymatic extract, including alginate, alginate-silica method 1 (Alg-Si M1), alginate-silica method 2 (Alg-Si M2) and silica sol-gel.

[b]The encapsulation efficiency was determined as the relative percentage of entrapped protein (mg) to that present in the washing solutions.

[c]The specific activity of laccase was expressed as µmol converted ferulic acid/mg protein/min.

[d]Total activity in µmol converted ferulic acid/min, was calculated, by multiplying the total entrapped protein by the specific activity.

[e]The residual laccase activity (%) was defined as the relative laccase specific activity (µmol converted ferulic acid/mg protein/min) of the encapsulated enzyme extract to that of the free one.

[f]Relative percentage standard deviation was defined as the standard deviation of triplicate trials divided by their respective means, multiplied by 100.

Lee and Mooney (2012) indicated that the increase in the viscosity of alginate was in concomitance with a decrease in the pH value, which in turn may affect the matrix porosity; these authors also reported that the highly viscous alginate preparations are often undesirable in processing and may lead to a post-gelling stiffness and damage risks, with the use of high shear forces that are applied during mixing.

Bou-Mitri (2013) reported that the encapsulation of the laccase enzymatic extract, from *C. hirsutus,* in calcium alginate beads resulted in an EE of 60.9% and a residual enzyme activity of 62.6%. However, Daássi *et al*. (2014) determined that the encapsulation of laccase, from *Coriolopsis gallica*, in calcium alginate beads, resulted in a relatively higher EE and residual enzyme activity. Phetsom *et al*. (2009) indicated that the alginate encapsulation of laccase in selected bivalent cations resulted in an EE, ranging from 40 to 60%.

3.4.2. Encapsulation of the laccase extract in alginate-silica hybrids

The results presented in Table 3.1 indicate that the encapsulation of laccase extract in alginate-silica hybrid by M1 and M2 methods, resulted in 53.5 and 70.7% EE, respectively, with a corresponding residual enzyme activity of 37.4 and 68.6%. In addition, the experimental findings (Table 3.1) also indicate a significantly ($P \leq 0.05$) higher EE for the alginate-silica M2 as compared to that for the alginate, which may suggest that the incorporation of silica into the alginate matrix improved its mechanical stability.

Coradin *et al*. (2003) suggested that the alginate-silica formation relies on the permeation and polymerization of the partially hydrolyzed silanol species within the alginate gel; the difference between M1 and M2 was in the approach used for the hydrolysis of the silica precursor (TEOS), where M1 involved the slow hydrolysis of TEOS by the guluronic and mannuronic acids of the sodium alginate, whereas in M2, a pre-hydrolyzed silica sol was used for preparing the alginate-silica hybrids. In terms of EE and residual laccase activity, the experimental results (Table 3.1) suggest that M2 could be a more reliable approach for the formation of alginate-silica hybrids. The higher protein leakage, obtained with M1, may have resulted from a longer incubation time of the alginate beads in TEOS. Likewise, a much lower residual laccase activity in the M1 matrix may be associated with the long-term exposure of the encapsulated laccase extract to

alcohol, a major by-product of the hydrolysis and condensation reactions of the silica precursors (Mohidem and Mat, 2012a).

Similar findings were reported by Yi *et al.* (2005) and Heichai-Segal *et al.* (1995), who suggested that the presence of water in the interstitial spaces of alginate matrix was either used in the hydrolysis of the precursor, or was expelled during the formation of the silica gel network. In addition, Yi *et al*. (2005) also indicated that the entrapped enzyme within the alginate would either interact with the silica gel or was released with the expelled water, resulting in a higher protein leakage.

The experimental findings shown in Table 3.1 are in agreement with those reported in the literature. Shao *et al.* (2009) indicated that the encapsulation of polyphenol oxidase in a hybrid gel of alginate-silica resulted in the enhancement of the enzyme stability. Likewise, Xu *et al.* (2007) reported that the encapsulation of yeast alcohol dehydrogenase (ADH), in alginate-silica hybrids, resulted in 21% less protein leakage as compared to that in the alginate matrix alone; these authors suggested that this difference was due to the inhibition of protein leakage by the silica film. Similarly, Lu *et al.* (2006b) determined a significantly reduced formate dehydrogenase leakage with the use of hybrid gels, as compared to with the pure alginate matrices. Xu *et al.* (2006a,b) also reported that a higher loss of protein from the alginate matrix was in concomitance with the increase in water loss; these authors further concluded that the alginate-silica matrix resulted in a more confined environment for the encapsulation of the enzymes.

3.4.3. Encapsulation of the laccase extract in silica sol-gel matrix

The experimental findings presented in Table 3.1 for the encapsulation of the enzymatic extract in silica sol-gel matrix indicated a significant ($P \leq 0.05$) enhancement of the encapsulated laccase activity (51.6 µmol/mg protein/min) as compared to that for the free one (16.3 µmol/mg protein/min). Furthermore, the sol-gel encapsulation of the enzymatic extract also resulted in a significantly ($P \leq 0.05$) higher EE (90.4%) and residual laccase activity (319.9%), as compared to the other investigated matrices.

The enhancement of the laccase catalytic activity for the encapsulated enzyme in the silica sol-gel was also reported by Mohidem and Mat (2009), who indicated that the sol-gel network around the entrapped enzyme provided an effective substrate diffusion and an improved enzymatic protein flexibility; these authors also reported that the hydrophilic characteristic of silica, aided in minimizing the partitioning of substrates or products between the matrix and the surrounding medium, resulting in an enhancement of the enzyme catalytic activity. Lloret *et al.* (2011) as well as Qiu and Huang (2010) reported a relatively high EE for the encapsulation of laccase in sol-gel matrix.

3.4.4. Catalytic properties of the encapsulated laccase extract

The kinetic studies for the free and the encapsulated laccase enzymatic extracts, in the alginate, alginate-silica (M1, M2) and silica sol-gel matrices were carried out, with the use of a wide range of FA concentrations (0-50 mM), at a constant temperature of 50°C and pH of 5.0. The Lineweaver-Burk plots of $1/v$ versus 1/[S] displayed a linear relationship for the free and the encapsulated enzyme extracts.

The experimental findings in Table 3.2 indicate that the encapsulated enzymatic extracts, in alginate as well as alginate-silica matrices M1 and M2, demonstrated relatively lower K_m of 0.23, 0.65 and 0.59 µM, respectively and also resulted in a significant decrease in their corresponding V_{max} of 20.39, 13.89, 17.98 µmol/mg/min, as compared to the free one, with K_m of 0.73 µM and V_{max} of 41.26 µmol/mg/min. The low K_m values for the encapsulated laccase extract may indicate a higher substrate affinity as compared to the free one. In addition, the catalytic efficiency (k_{cat}) for the alginate encapsulated laccase extract was also relatively higher (89.87 min^{-1}) than that for the free one (56.88 min^{-1}). Furthermore, the k_{cat} for the alginate-silica matrices M1 and M2 was 20.07 and 30.55 min^{-1}, respectively. On the contrary, the silica sol-gel encapsulated laccase extract resulted in a relatively higher K_m value of 4.55 µM and significantly ($P \leq 0.05$) enhanced V_{max} and k_{cat} of 311.10 µmol/mg/min and 63.70 min^{-1}, respectively, as compared to that for the free enzyme extract.

Zhou *et al.* (2010) reported that the K_m and V_{max} value for the alginate chitosan beads encapsulated yeast ADH, was higher and lower, respectively, than that for the free one.

Table 3.2. Michaelis-Menten kinetic parameters for the encapsulated laccase extract in alginate, alginate-silica M1 (Alg-Si M1), alginate-silica M2 (Alg-Si-M2) and silica sol-gel matrices.

Encapsulation matrix	K_m[a]	V_{max}[b]	k_{cat}[c]	k_{cat}/K_m[d]
Free	0.73 (8.02)[e]	41.26 (3.21)	56.88 (4.82)	78.68 (12.82)
Alginate	0.23 (2.50)	20.39 (2.16)	89.87 (0.34)	396.22 (2.84)
Alg-Si M1	0.65 (6.09)	13.89 (3.90)	20.07 (2.68)	31.20 (11.99)
Alg-Si M2	0.59 (7.49)	17.98 (11.62)	30.55 (4.19)	52.06 (3.36)
Sol-gel	4.55 (5.16)	311.10 (6.27)	63.70 (2.57)	14.02 (5.22)

[a]The Michaelis constant K_m was expressed in µM.

[b]V_{max}, the apparent maximum velocity of laccase was defined as µmol converted substrate/mg encapsulated or free protein/min.

[c]The catalytic efficiency, k_{cat} was defined as the ratio of V_{max}/K_m and was expressed in min^{-1}.

[d]The specificity constant was expressed as the ratio of k_{cat}/K_m.

[e]Relative standard deviation (%) was calculated by means of triplicate trials.

Similarly, Lu *et al.* (2007) indicated a higher K_m value for the encapsulated laccase in alginate-chitosan hybrids, as compared to that for the free one. Rekuc *et al.* (2009) suggested that the rate of enzymatic reactions, in siliceous mesoporous cellular foams, depends on the rate of substrate transfer and the characteristic intrinsic activity of the enzyme; these authors also indicated that the low affinity of the silica gel encapsulated enzyme may be due to diffusional limitations within the matrix, where the lower enzyme activity at higher substrate concentrations could be a result of product accumulation at the surface of the gel, hence promoting mass transfer limitations. Likewise, Asgher *et al.* (2012) reported that the encapsulation of laccase, from *Pleurotus ostreatus*, in sol-gel matrix exhibited higher K_m and V_{max} values as compared to those for the free one. Lloret *et al.* (2011) also reported a lower enzyme substrate affinity for the encapsulated laccase and suggested that it may be due to the diffusional limitations inside the matrix.

3.4.5. Protein leakage profile of the encapsulated laccase extract

The protein release profiles for the selected encapsulation matrices of laccase extract are shown in Figure 3.1. These experimental findings indicate that the alginate, alginate-silica M1 and M2 as well as silica sol-gel, resulted in 25.1, 35.3, 5.1 and 0.4% protein leakage, respectively, at the end of 1 h of storage time, whereas this leakage was 46.3, 51.9, 18.5 and 12.3%, respectively at the end of 6 h. In addition, at the end of 12 h of storage time, the alginate, alginate-silica M1, M2 and sol-gel matrices demonstrated, respectively, 50.3, 54.9, 20.4 and 15.3% protein leakage, whereas this leakage was 56.8, 64.6, 26.8 and 20.6%, after 24 h of storage.

The results (Fig. 3.1) also indicate an immediate release of protein for both alginate and alginate-silica M1 matrices, in the first 6 h of storage, as compared to that for the sol-gel and the alginate-silica M2 matrices. The experimental findings suggest a relatively higher degree of diffusion of the enzyme extract for the alginate and alginate-silica M1 matrices. Overall, the experimental findings suggest that the incorporation of the silica into the alginate, using the M2 approach, could improve the matrix stability and hence resulted in a reduced enzyme leakage.

Khani *et al.* (2006) reported certain protein leakage for both alginate encapsulated laccase and glucose oxidase, with a reduced diffusion rate in the alginate matrix containing carbon; this may be due to the irreversible enzyme binding to the matrix as a result of the ionic and hydrophobic

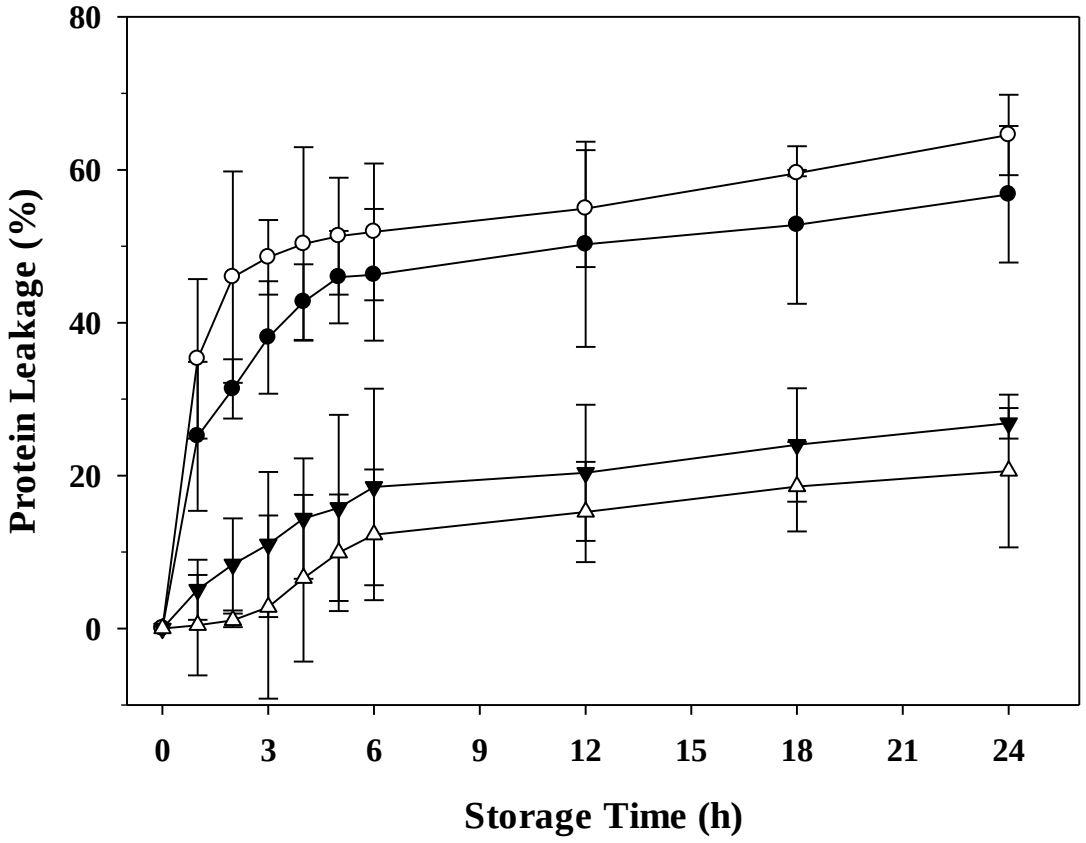


Figure 3.1. Protein release profile of the encapsulated laccase extract in alginate (●), alginate-silica M1 (○), alginate-silica M2 (▼) and silica sol-gel matrix (Δ).

interactions. Yi *et al.* (2005) indicated the protein release profile for alginate encapsulated chlorophyllase and concluded that the high matrix porosity resulted in a relatively important leakage.

3.4.6. Re-usability of the encapsulated laccase extract

The re-usability of the laccase extract, encapsulated in selected matrices, was investigated for six continuous reaction cycles (Fig. 3.2). The relative residual laccase activity for the encapsulated enzyme extract, after six cycles, in alginate, alginate-silica M1, M2 and sol-gel, was found as 71, 179.3, 49.6 and 49.7%, respectively. With the exception of alginate-silica M1, the results (Fig. 3.2) indicate that the residual laccase activity for the encapsulated enzyme extract decreased with its continuous re-usability. The results also demonstrate that the relative residual laccase activity for the alginate and the sol-gel encapsulated enzymatic extract was relatively high, with 71 and 82%, respectively, until the fifth reaction cycle; these findings indicate an enhancement in the enzyme re-usability. On the contrary, the relative residual laccase activity in the alginate-silica M2 matrix dropped to almost 40%, by the end of 5 reaction cycles; this sudden decrease could be associated to a higher protein leakage.

The results (Fig. 3.2) for the re-usability of the encapsulated laccase extract in the alginate-silica M1 matrix was quite unusual, with a relatively higher residual enzyme activity than that obtained in the first cycle. The experimental findings (Table 3.1 and Fig. 3.1) for the alginate-silica M1 could indicate the high porosity of the matrix and its inability to retain the enzyme for a longer period of time. Despite the higher protein leakage, the increased laccase activity in the successive cycles could be a result of a much stronger and irreversible association of the enzyme extract with the support matrix (De Hoog *et al.*, 2010). Zhang *et al.* (2016) suggested that although a strong binding of the enzyme with the silica matrix may lower its specific activity, it can also maximize its re-usability. Hence, this could also explain the results (Table 3.1), that even though there was no significant difference in the EE for the alginate, alginate-silica M1 and M2, the residual laccase activity was the lowest for the alginate-silica M1. Similarly, De Hoog *et al.* (2010) reported that lipase-containing polymersomes, immobilized in hydrogel, resulted by an increase in its activity and re-usability in the subsequent reaction cycles; these authors also indicated that this increase in the enzyme activity was due to the pore expansion,

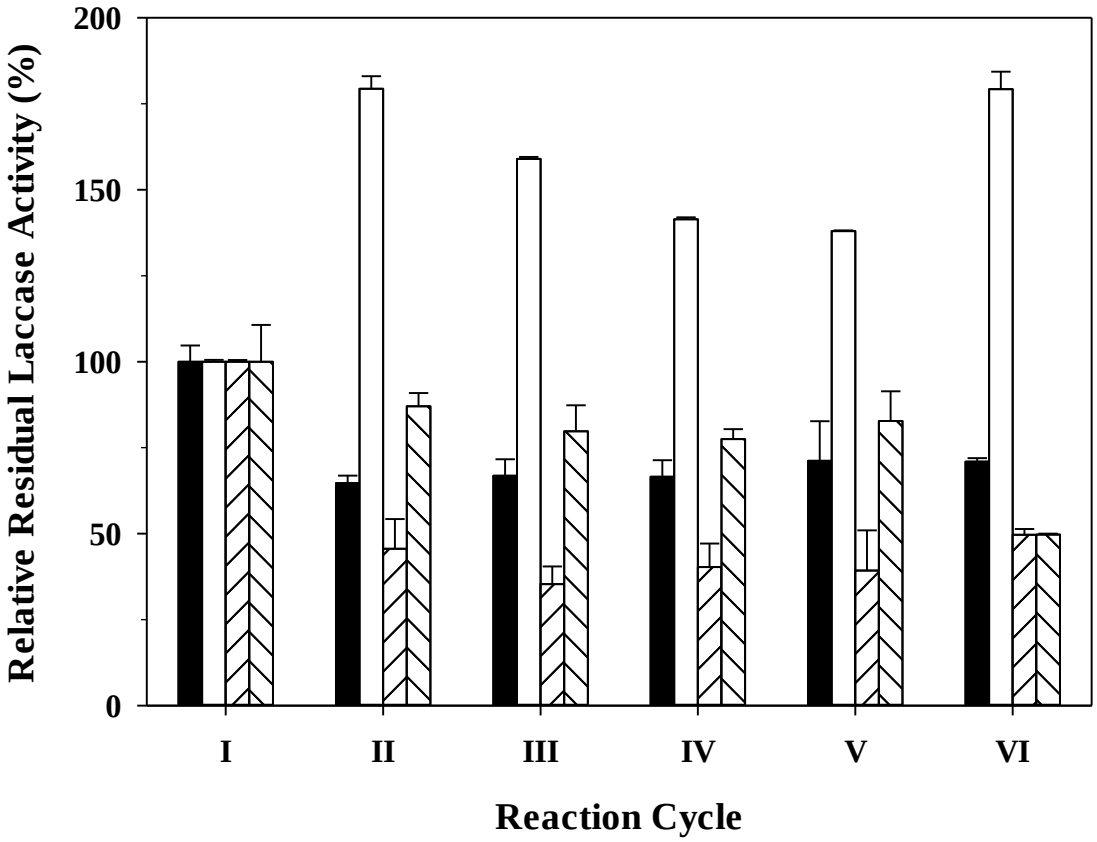


Figure 3.2. Re-usability of the encapsulated laccase extract in alginate (■), alginate-silica M1 (□), alginate-silica M2 (▨) and silica sol-gel matrices (▧). Reaction conditions: sodium acetate buffer (0.1 M, pH 5.0), 50°C, 0.03 M ferulic acid substrate. The relative residual laccase activity (%) was defined as the specific activity of the encapsulated enzyme extract at a defined cycle as compared to the specific activity at time 0. The relative standard deviations are between 0.2 to 12%.

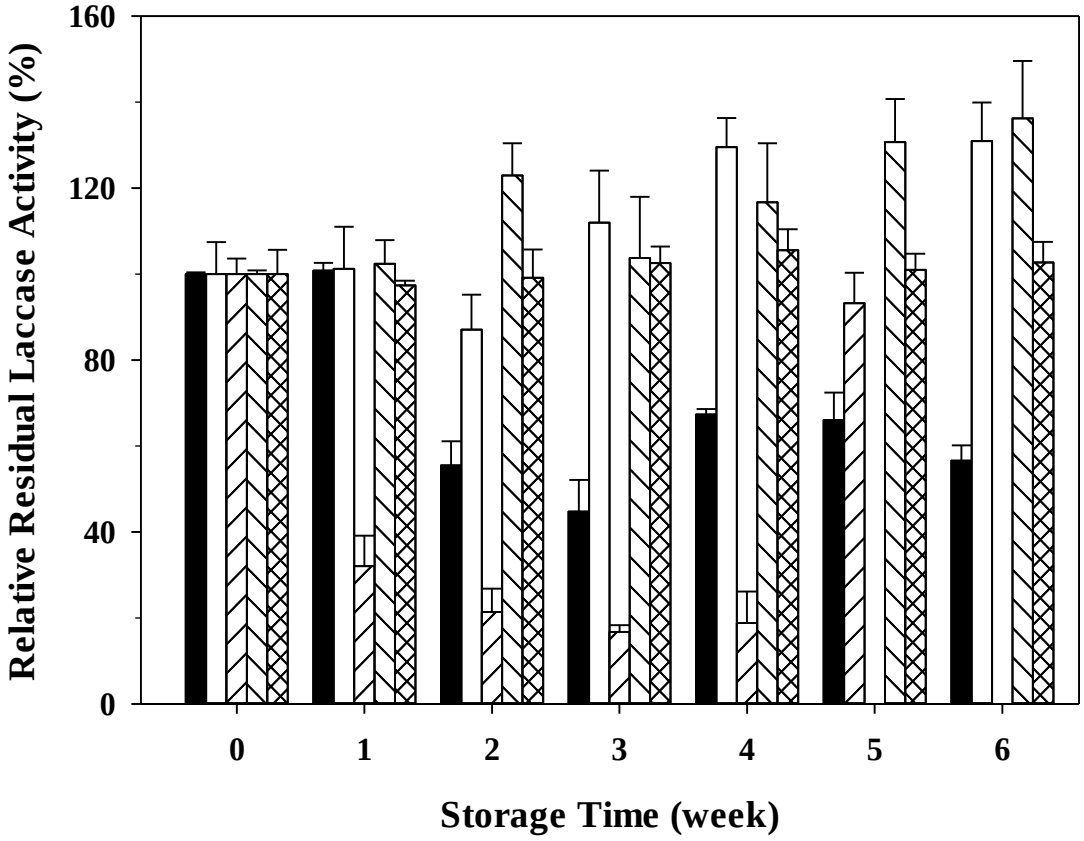


Figure 3.3. Storage stability of enzymatic laccase extract at 4°C in alginate (□), alginate-silica M1 (▨), alginate-silica M2 (▧) and sol-gel (▩) matrices, in comparison to the free enzyme extract (■). The relative residual laccase activity (%) was defined as the specific activity of the encapsulated or free enzyme extract after storage at 4°C to that at week 0. All results are means of triplicate trials and the relative standard deviations vary between 0 to 14%.

resulting from the dimethyl sulfoxide which was used for the solubilization of the substrate. Li *et al.* (2011) reported that the immobilization of horseradish peroxidase in silica glass resulted in a 35% decrease in the enzyme activity after the first three reaction cycles, but with a relatively stable enzyme activity in the subsequent ones. Patel *et al.* (2014) reported that laccase entrapped in silica nanoparticles, showed a relatively high residual enzyme activity after 10 reaction cycles. Yamak *et al.* (2009) indicated that the re-usability of laccase, encapsulated in different alginate matrices, demonstrated a relatively high residual enzyme activity for 10 continuous cycles.

3.4.7. Storage stability of the encapsulated laccase extract

The stability of the laccase activity of the free and the encapsulated enzymatic extracts was determined at 4°C, over a period of 6 weeks. The encapsulated laccase enzymatic extract in alginate, alginate-silica M2 and sol-gel matrix, as presented in Figure 3.3, showed a relative residual enzyme activity of 131, 136 and 103%, respectively, as compared to 56% for the free one. The enzymatic extract, encapsulated in alginate-silica M1, demonstrated a negligible laccase activity after 4 weeks of storage.

Bou-Mitri (2013) reported that the residual activity for the alginate encapsulated laccase, at -80°C, for 6 weeks of storage, was 70%. Xu *et al.* (2006a) reported that the residual ADH activity for the alginate, alginate-silica gel and alginate-silica sol-gel matrices, was respectively, 58, 78 and 76%, after a period of 30 days storage. Lloret *et al.* (2011) investigated the storage stability of silica sol-gel encapsulated laccase at 4°C and at room temperature, where it was relatively stable for 3 months, under both conditions.

3.5. Conclusion

The present study suggests that the catalytic activity and stability of the *Coriolus hirsutus* laccase enzymatic extract could be improved by its encapsulation in silica sol-gel matrices. The experimental findings indicate that the modification of the pH for the initial pre-gel alginate solution could result in an enhancement of the residual laccase activity in the alginate matrix; the matrix demonstrated not only an appropriate encapsulation efficiency and residual enzyme activity, but also showed adequate storage stability. In addition, the hybridization of alginate with silica appeared to be a promising approach for the encapsulation of laccase extract in

organic polymers. Overall, the use of different immobilization supports and approaches, resulted in an encapsulated laccase of higher catalytic activity, stability and re-usability, which may promote its use in a wide range of biotechnological applications.

CONNECTING STATEMENT TO CHAPTER IV

The encapsulation of a laccase enzymatic extract in selected matrices was reported in Chapter III. Chapter IV involves the screening trials for the encapsulation of a laccase enzymatic extract in a silica sol-gel matrix. In order to determine the most significant effects on the residual laccase activity and the encapsulation efficiency, selected parameters, including gel drying time, gel aging time, water to silane molar (*r*) ratio, HCl content and enzymatic protein loading were investigated.

CHAPTER IV

SCREENING TRIALS FOR THE ENCAPSULATION OF LACCASE ENZYMATIC EXTRACT IN SILICA SOL-GEL

4.1. Abstract

The effects of selected process conditions for the sol-gel encapsulation of the laccase enzymatic extract, obtained from *Coriolus hirsutus*, were investigated. Screening trials were carried out to identify the parameters having the most pertinent effects on the encapsulation efficiency (EE) and the residual laccase activity. These parameters included water/silane molar ratio (*r*), HCl content and protein loading, for the pre-gel silica sol as well as the required time for gel drying and for aging during the sol-gel process. The experimental findings indicated that a sol-gel drying time of over 6 h resulted in a complete loss of laccase catalytic activity, while an increase in the gel aging time led to an enhancement of the residual enzyme activity. Except for *r*, the investigated parameters demonstrated no significant effect on the EE of the sol-gel encapsulated enzymatic extract. Overall, the encapsulation of laccase extract in the sol-gel matrix resulted in an enhancement of its catalytic activity, where its highest residual activity (349%) was obtained with an *r* value of 4, an HCl content of 4 µmol and a protein loading of 1 mg/mL, using, respectively, 6 and 24 h of drying and aging times.

4.2. Introduction

Laccases (EC 1.10.3.2) are blue multi copper oxidases (MCO) that catalyze the oxidation of various aromatic as well as non-aromatic substrates, with the concomitant reduction of molecular oxygen (Rekuć *et al*., 2010). Laccases are ubiquitous in nature and have attracted considerable attention for their potential applications in various biotechnological processes, including detoxification, bio-remediation, bio-sensing, bio-transformations of natural compounds, pulp and paper, nanobiotechnology, cosmetics and textiles (Piscitelli *et al*., 2010; Rekuć *et al*., 2010). The growing interest in laccase is principally due to their low substrate specificity and their ability to catalyze various compounds, including alkenes, aminophenols, polyphenols, polyamines as well as lignin-related compounds (Gasser *et al*., 2014).

In general, the limitation of an efficient use of laccase in industrial applications is often due to the poor enzyme stability as well as difficulties in its recovery and its reusability. The optimum pH of laccases ranges between 5 to 7 and the optimal temperature range has been reported to be between 20 to 85°C (Gasser *et al*., 2014). Nevertheless, these limitations can be overcome by the use of an effective method of encapsulation of laccase, which could ultimately widen its scope in continuous biotechnological processes (Rekuć *et al*., 2010; Gasser *et al*., 2014). Numerous studies have investigated various methods and supports for the immobilization of laccase (Duran *et al*., 2002; Fernández-Fernández *et al*., 2013; Guzik *et al*., 2014). In particular, the encapsulation of laccase in silica sol-gel has been reported to produce an efficient biocatalyst, with an enhancement of the enzyme stability and activity (Mohidem and Mat, 2009; Mohidem and Mat, 2012a,b). Moreover, sol-gel technology offers various other advantages, including chemical inertness and mild processing conditions that could maintain the native enzyme/protein conformation, non-toxicity, optical transparency and a tuneable porosity (Jin and Brennan, 2002; Lloret *et al*., 2011). Despite all these advantages, sol-gels are usually brittle and are often susceptible to cracking and have also been known to undergo excessive volume shrinkage. In addition, enzyme substrate inhibition can also occur inside the encapsulation matrix, which may be due to the compact structure of the silica gel network (Jin and Brennan, 2002).

Most of the previous published work was related to the use of commercial enzyme extract obtained from microbial sources other than *Coriolus hirsutus*, including that from *Trametes* species and *Miceliophthora thermophilca* or from unknown microbial sources (Mohidem and Mat, 2012a,b; Qiu and Huang, 2010; Lloret *et al*., 2011). In addition, the laccase used throughout our study was an ultra-filtrate enzyme extract, obtained without the use of any commercial diluent such as that reported by Mohidem and Mat (2012b).

Moreover, investigating the various process parameters is of significant importance for the development of an efficient method for the encapsulation of laccase extract in silica sol-gel. Indeed, Dickson and Ely (2011) indicated that the sol-gel encapsulation of enzymatic extracts and biological components has not been fully understood from the mechanistic principles, due

to the complexity of the sol-gel chemistry and the variety of materials that could be encapsulated in these matrices.

The aim of the current research work was to investigate the effects of selected process parameters to screen and to identify the ones having the most significant impact on the silica sol-gel encapsulation of a laccase enzymatic extract. The effects of the gel drying time as well as aging time, water to silane molar ratio, HCl content and protein loading were determined on the residual laccase activity and the EE.

4.3. Materials and Methods

4.3.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). FA and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Tetraethyl orthosilicate (TEOS) (98% purity) and all organic reagents of HPLC grade or higher were obtained from Fischer Scientific (Fair Lawn, N. J.). Ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). All reagents and buffers were prepared in deionized water, using Milli Q plus (Millipore).

4.3.2. Production and recovery of laccase enzymatic extract from Coriolus hirsutus

The production of *Coriolus hirsutus* biomass and the recovery of the enzymatic extract were carried out according to the procedure described by Taqi (2012). All protocols were performed at 4°C and the enriched enzymatic extract was lyophilized in the presence of mannitol as an additive (2.5% w/v); the enzyme powder was then stored at -80°C for further analysis.

4.3.3. Standard protocol for the encapsulation of laccase enzymatic extract in silica sol gel

The sol-gel encapsulation of the laccase enzymatic extract was carried out with a modification to the method described by Mohidem and Mat (2012a,b). Laccase extract (1 mg protein/mL TEOS sol) was suspended in 1 mL potassium phosphate buffer (0.01 M, pH 6.0) and added to a pre-hydrolyzed silica sol containing 2.5 mL tetraethylorthosilicate (TEOS), 0.4 mL deionised water and 0.1 mL of 0.04 M HCl (4 µmol). The sol-gelation was facilitated by an immediate addition of 2 mL potassium phosphate buffer (0.4 M, pH 7.0), with continuous agitation until

the onset of gelation (1-3 min). The resulting gel was aged for 24 h at 4ºC, followed by crushing, then washed and filtered using Whatman filter paper (No. 1). The wet gel was then lyophilized for 6 h at -42°C and stored under refrigeration to maintain its catalytic activity. All washing solutions were collected to analyze their protein content.

4.3.4. Protein determination

The protein content of the free enzyme extract and the washing solutions was determined as per a modification to the Lowry method (Hartree, 1972), where bovine serum albumin (BSA) was used as a standard for the calibration curve.

4.3.5. Enzyme activity

The enzyme activity assay for both free and encapsulated laccase was carried out according to Bou-Mitri (2013). A sample of free or encapsulated laccase was suspended in sodium acetate buffer (0.1 M, pH 5.0), and the enzyme activity was measured by following the oxidation of FA (0.03 M) as a substrate. The decrease in the absorbance was measured at 320 nm in an UV-vis spectrophotometer (Beckman Instruments Inc., San Raman, CA), at regular time intervals (0 to 10 minutes). All reactions were carried out at 50°C with continuous stirring at 150 rpm, where the assays were performed in triplicate trials and were run simultaneously with a blank reaction containing everything except the enzyme. The specific activity was defined as µmol of converted ferulic acid/mg protein/min.

4.3.6. Encapsulation efficiency (EE) and residual laccase specific activity (%)

The encapsulation efficiency (EE) was defined as, the difference between the total initially added protein to the silica sol, and the protein determined in the recovered washing solutions, respectively. The EE and the enzyme residual specific activity were calculated as follows:

$$\text{Encapsulation efficiency (EE) (\%)} = (C_0 V_0 - C_f V_f) / (C_0 V_0) * (100) \quad \text{(I)}$$

$$\text{Enzyme residual activity (\%)} = (SA_{imm}) / (SA_{free}) * (100) \quad \text{(II)}$$

where, C_0 and C_f represent the initial loaded protein and the final protein concentration in the washing solutions, while V_0 and V_f were the initial and final volumes of the sol and washing solutions, respectively. SA_{free} and SA_{imm} was the specific activity of the free and encapsulated

laccase enzymatic extract, correspondingly and was defined as µmol converted ferulic acid/mg/protein/min.

4.3.7. Screening single factor experiments

Screening trials were performed to identify and to determine the parameters having the most significant effects on the EE and the residual laccase activity in silica sol-gel matrix.

4.3.7.1. Gel drying time

The sol-gel encapsulated laccase was prepared according to the standard protocol described previously, except that the prepared gels were freeze-dried between, 0 to 15 h, after aging for a minimum of 6 h and assayed for the enzyme activity. The water activity of the dried gels (a_w) was also measured at 25°C by a Novasina AW SPRINT TH-500 system (Axair Ltd.; Pfaffikon, Switzerland), using humidity reference points.

4.3.7.2. Gel aging time

The sol-gel entrapped laccase was prepared as per the standard protocol as described earlier, while aging the gel at varying time intervals from, 6 to 24 h at 4°C.

4.3.7.3. Water to silane molar ratio (r)

The effect of *r* was determined by increasing the volume of water from 0.4 to 2.0 mL in the pre-gel silica sol containing, 0.1 mL of 0.04 M HCl and 2.5 mL of TEOS, to obtain r between 2.0 to 10.0.

4.3.7.4. HCl content

The effect of varying the initial HCl content in the TEOS sol was investigated within a range of 2.0 to 8.0 µmol, using the standard conditions of 0.4 mL water and 2.5 mL of TEOS.

4.3.7.5 Protein loading

The effect of protein loading was investigated by encapsulating the enzymatic laccase suspension of varying protein concentrations (0 to 1.5 mg protein/mL TEOS sol), using the standard sol-gel protocol, described previously.

4.3.8. Statistical analysis

All data were expressed by means of at least three independent trials and their respective standard deviations (SD). The relative standard deviation (RSD) was defined as the SD divided by the mean multiplied by 100. Correlation analyses were performed using Microsoft-Excel (Microsoft) and the data was analyzed by PROC ANOVA with a post-hoc analysis using Tukey's honest significance test; values where $P \leq 0.05$ were considered significant.

4.4. Results and Discussion

4.4.1. Effects of sol-gel drying time on the residual laccase activity and EE

Table 4.1 summarizes the experimental findings, obtained for the effect of lyophilization time on the residual enzymatic activity and the EE for the silica sol-gel encapsulated laccase. Although the results indicated no significant difference in the EE of laccase extract, the residual enzyme activity decreased with the increase in the gel drying time; a complete loss in its catalytic activity was obtained when the gel was dried over a period of 6 h. In addition, Figure 4.1 indicates that a water activity (a_w) of 0.92, 0.88, 0.72, 0.16 and 0.14 was obtained, respectively, after drying times of 3, 6, 9, 12 and 15 h.

Petersson *et al.* (2007) suggested that a_w is an important parameter for the enzyme activity. Although, the amount of required water can vary for different enzymes, a certain limit is necessary to retain their catalytic activity (Zaks and Klibanov, 1985; Elfman-Borjesson and Harrod, 1999). Table 4.1 shows that the sol-gel encapsulated laccase extract, dried for up to a period of 6 h, may have retained its catalytic activity as a result of the presence of an adequate amount of water. Similar findings were reported by Yi *et al.* (2006), where the sol-gel encapsulated chlorophyllase demonstrated negligible enzyme activity after 12 h of drying time; it was suggested that the loss in the enzyme activity was due to the removal of the essential bound water, which may have caused detrimental conformational changes to the enzyme or pore shrinkage and gel stiffness. Likewise, Vila-Real *et al.* (2010) indicated that an increase in the sol-gel aging and drying time led to an enhanced gel density and strength; however, such increase resulted in gel shrinkage, which may have promoted mass-transfer limitations. Based on the experimental findings (Table 4.1), a drying time of 6 h was used throughout the present study.

Table 4.1. Effect of the sol-gel drying time on the encapsulation efficiency and the residual enzyme activity of laccase obtained from *Coriolus hirsutus*, assayed with ferulic acid at 50°C and pH 5.0

Drying time (h)	Encapsulation efficiency (%)[a]	Residual laccase activity (%)[b]
0	90.64 (0.65)[c]	1151.77 (2.69)[c]
3	89.49 (0.66)	406.07 (5.70)
6	88.46 (0.75)	186.51 (3.40)
9	88.59 (0.25)	n.d.[d]
12	90.42 (0.46)	n.d.
15	90.97 (0.53)	n.d.

[a]The encapsulation efficiency was defined as the relative percentage of the encapsulated protein (mg) in the sol-gel to that of the initial loaded protein of the free enzyme extract.

[b]The residual laccase activity (%) was defined as relative percentage of the specific activity (µmol converted ferulic acid/mg protein/min) of the sol-gel encapsulated laccase to that of the free one.

[c]Relative percentage standard deviation was determined by dividing the standard deviation between triplicate trials by the mean, multiplied by 100.

[d]Not detected.

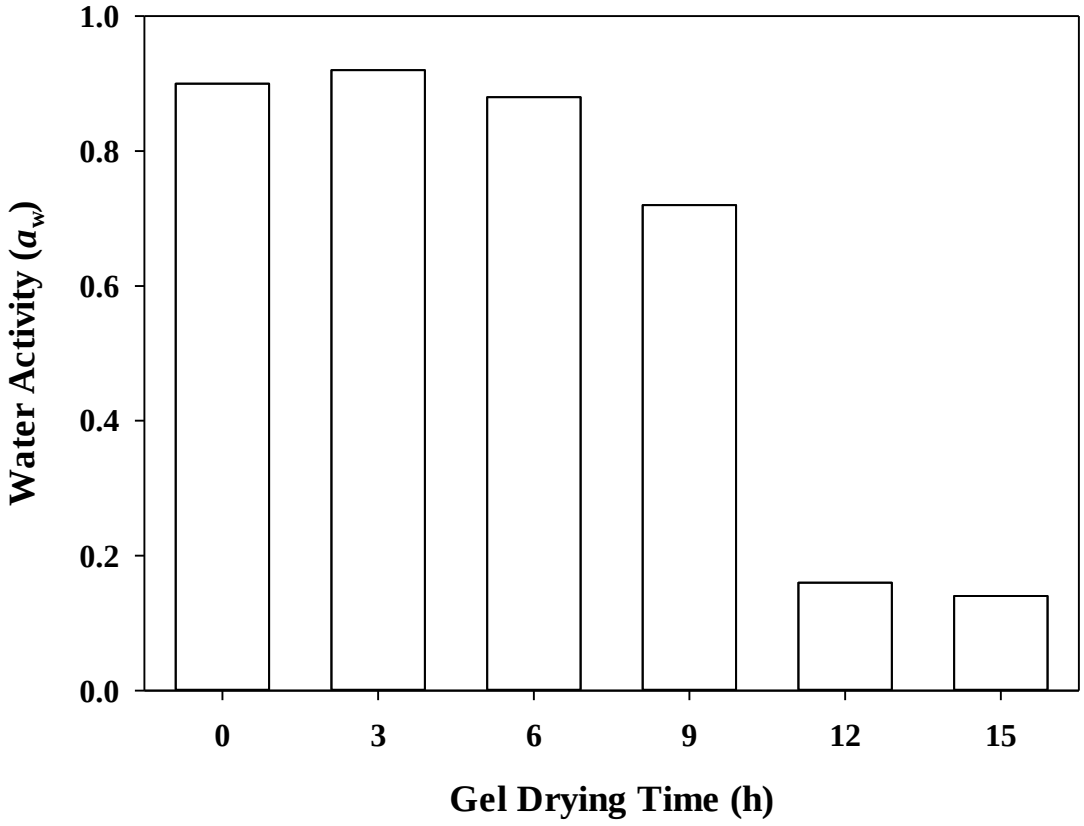


Figure 4.1. Effect of sol-gel drying time on the water activity (a_w) (□) of the encapsulated laccase. The a_w measurements of the freeze-dried gels were determined at 25°C, using the humidity reference points.

4.4.2. Effects of sol-gel aging time on the residual laccase activity and EE

Figure 4.2 demonstrates the effects of gel aging time on the residual laccase activity and EE of the sol-gel encapsulated laccase extract. The use of sol-gels aged for 6, 9, 12, 18 and 24 h resulted in a residual laccase activity of 187, 191, 227, 272 and 349%, respectively, with an EE ranging from 88 to 92%. The experimental findings suggest that an increase in the gel-aging time resulted by a concomitant increase in the residual laccase activity.

Desimone *et al.* (2008) investigated the immobilization of urease in sol-gel matrix and suggested that longer aging times promoted the condensation of the silica gel network, hence resulting in an increased mechanical strength and internal viscosity as well as its catalytic activity. Similarly, Vila-Real *et al.* (2010) reported that the optimum aging time for the sol-gel encapsulation of naringinase was 4 to 14 h and suggested that aging was a critical step for the strengthening of the silica gel network; however, an excessive aging time could result in an increase in pore shrinkage, gel cracking and mass-transfer limitations. Yi *et al.* (2006) also reported an enhancement of the catalytic activity of sol-gel encapsulated chlorophyllase extract, with an increase in the gel aging time; these authors suggested that the use of a longer aging time resulted in a stronger silica gel structure which reduced gel shrinkage, upon drying. Although other authors (Desimone *et al*., 2008; Chang *et al*., 2010; Qiu and Huang, 2010) have reported the use of longer aging times, a maximum of 24 h was investigated for practical purposes, and hence used throughout this study.

4.4.3. Effects of water to silane molar ratio (r) on the residual laccase activity and EE

The effects of *r* on the residual enzyme activity and EE of laccase extract were investigated by increasing the water content in the pre-gel silica (TEOS) sol. The results presented in Figure 4.3 indicate that the residual laccase activity increased slightly, from 349 to 395%, when the *r* was increased, respectively, from 2 to 4. Alternatively, a significant ($P \leq 0.05$) decrease in the laccase activity was demonstrated at *r* of 6, 8 and 10 with a residual enzyme activity of 300, 243 and 241%, respectively. In addition, the sol-gel encapsulation of laccase extract with *r* value 2 and 4 resulted in 92 and 87% EE, which was relatively higher than that obtained with 6 (81%), 8 (79%) and 10 (80%) *r* values. The results suggest that the decrease in laccase activity with an *r* value above 4 could be due to an increase in the enzyme leakage or due to an enhanced enzyme

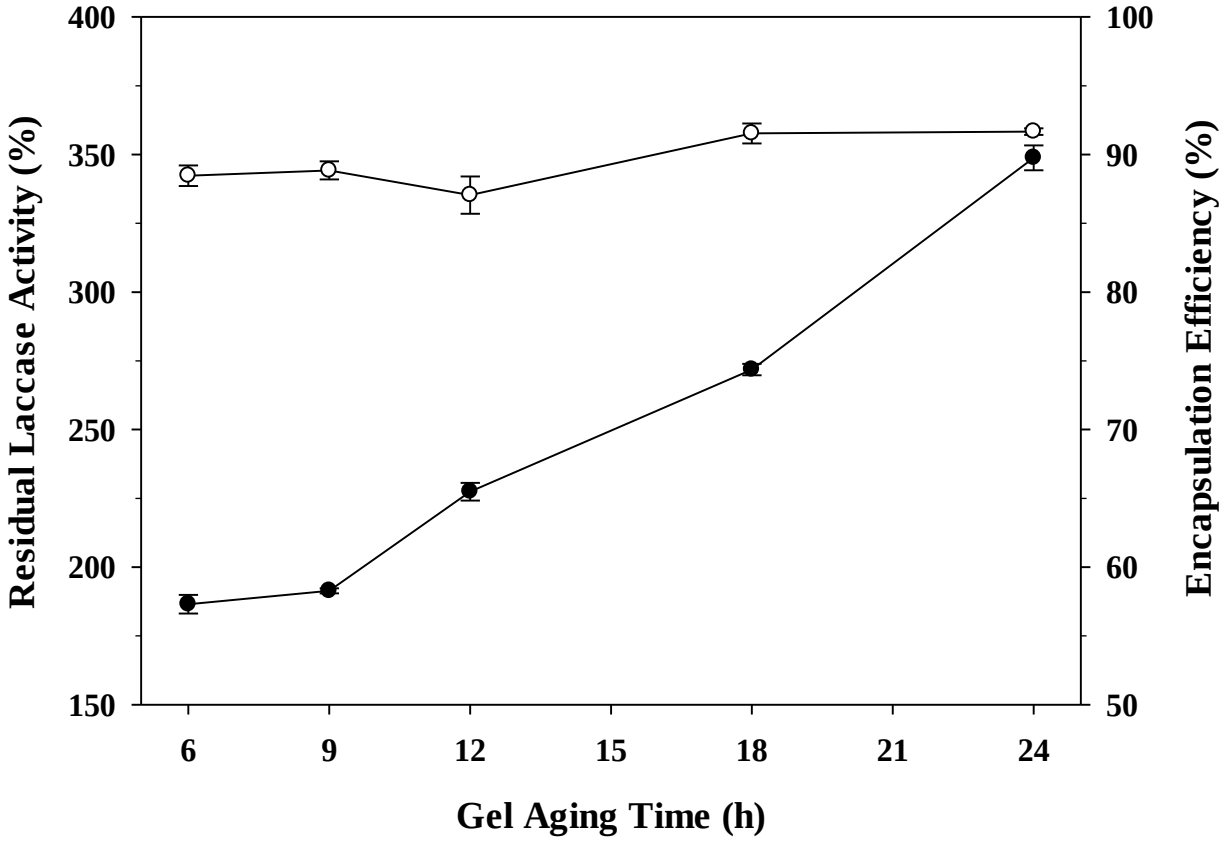


Figure 4.2. Effect of various gel aging times on the residual enzyme activity (●) and the encapsulation efficiency (○) of laccase entrapped in silica sol-gel matrix. Encapsulation conditions: water to silane ratio (2), 4 µmol HCl, 1 mg/mL protein and 6 h gel drying time. All results represent means of triplicate trials, with relative standard deviation of 0.2 to 9%.

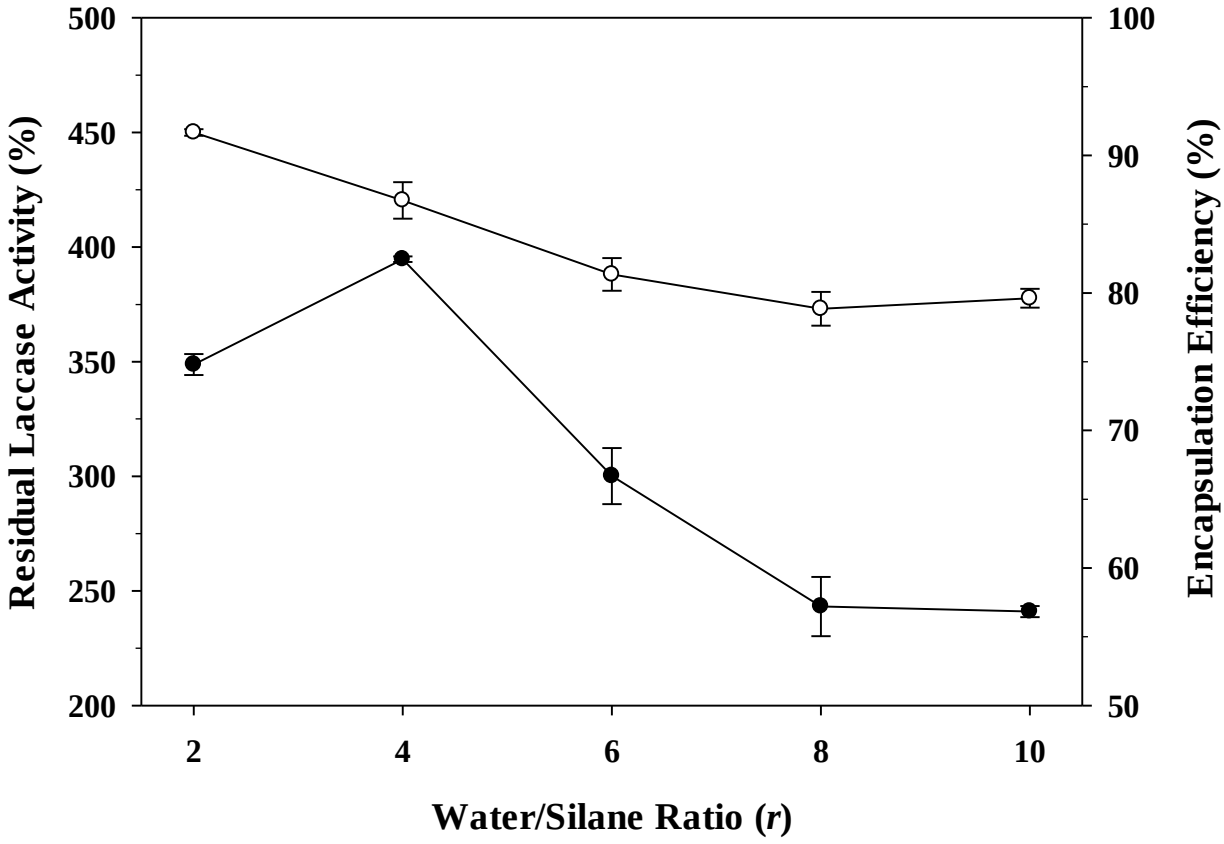


Figure 4.3. Effect of water to silane ratio on the residual enzyme activity (●) and the encapsulation efficiency (○) of laccase, using 4 μmol HCl, 1 mg/mL protein, 6 h of gel drying time and 24 h of aging time. All results are means of triplicate trials, with relative standard deviation of 0.2 to 13.0%.

mobility, which could promote its interaction with the silica and hence may have resulted in protein unfolding (Desimone *et al*., 2008). The experimental findings (Fig. 4.2) are in agreement with those reported in the literature. Hu *et al.* (2013) reported a decrease in the enzyme activity and EE of sol-gel encapsulated lipase, with r >15; the use of lower r values led to an enzyme agglomeration and alcohol condensation, thereby resulting in a decrease in enzyme activity, whereas higher r values led to a relatively lower EE. Similar findings were reported by Yang *et al.* (2009) who indicated that increasing the r value to a certain limit resulted in an initial increase in the enzyme activity of the encapsulated lipase; however, a further increase in water content resulted in a decrease in both enzyme activity and EE.

4.4.4. Effects of HCl content on the residual laccase activity and EE

The effects of HCl content in the pre-gel TEOS sol on the EE and the residual laccase activity were investigated (Fig. 4.4). With an acid content lower or higher than 4 µmol, the experimental findings showed a decrease in the residual laccase activity; however, there was no significant (P > 0.05) difference between the residual enzyme activity with HCl content of 2 (309%), 3 (308%), 4 (349%) and 6 µmol (345%). In addition, the acid content had no significant effect on the EE of the encapsulated laccase extract.

Rao and Bhagat (2004) indicated that an increase in the acid concentration during the preparation of the silica aerogel resulted in a decrease in the gelation time and particle pore size, with an increased gel density and volume shrinkage upon drying; these authors also reported a cracking of the gel at higher acid concentrations (> 0.001 M). Likewise, Huang *et al.* (1999) also suggested that the increase in HCl concentration resulted in shorter gelation times, whereas higher water content led to slower ones. The decrease in the residual laccase activity at higher acid contents may be due to an increase in the gel shrinkage upon drying (Rao and Bhagat, 2004). On the contrary, at a lower acid concentration, the increase in the gelation time and therefore the long-term exposure of the enzyme to the surrounding ethanol may have contributed towards a relatively lower residual laccase activity (Dickson and Ely, 2011).

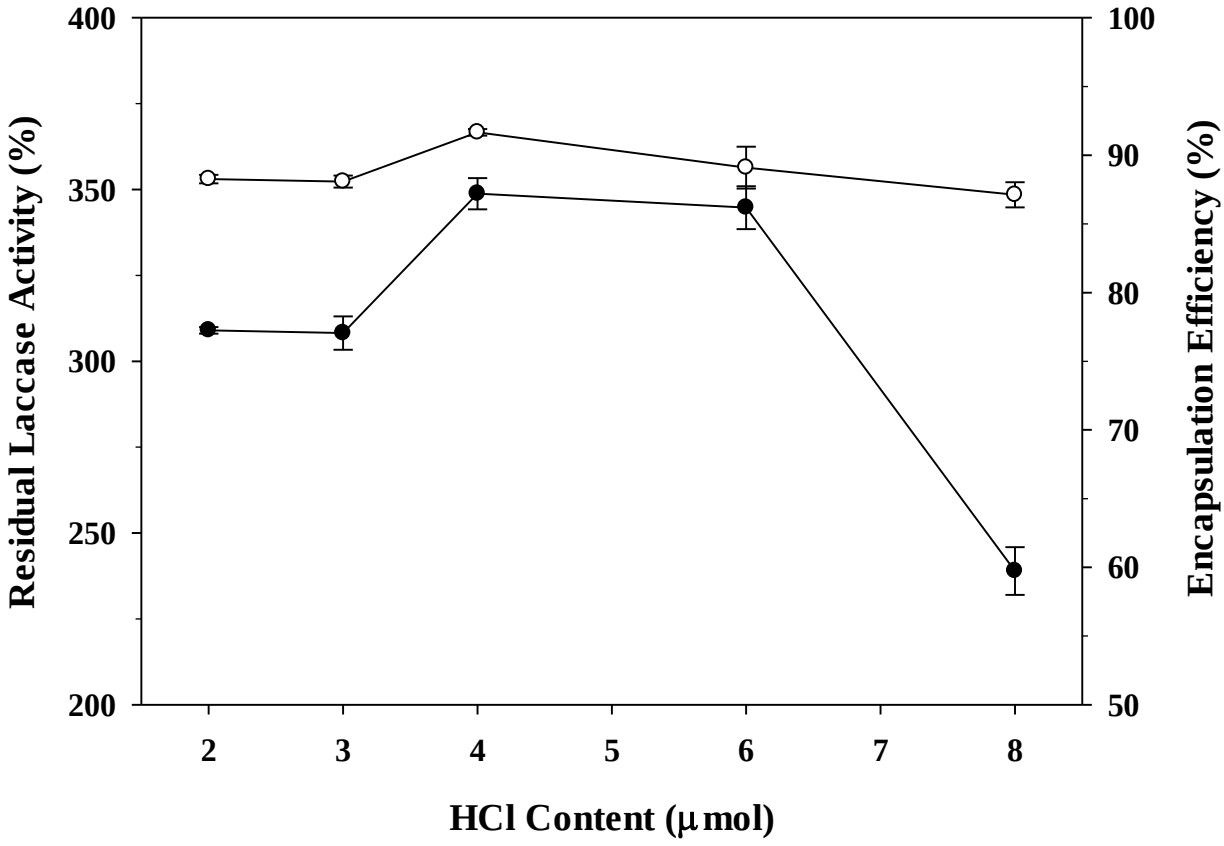


Figure 4.4. Effect of acid (HCl) content added to the Tetraethylorthosilicate (TEOS) on the residual enzyme activity (●) and the encapsulation efficiency (○) of sol-gel encapsulated laccase. Encapsulation conditions: water to silane ratio (2), 1 mg/mL protein, 6 h of gel drying time and 24 h of aging time, with relative standard deviation of 0.2 to 7.0%.

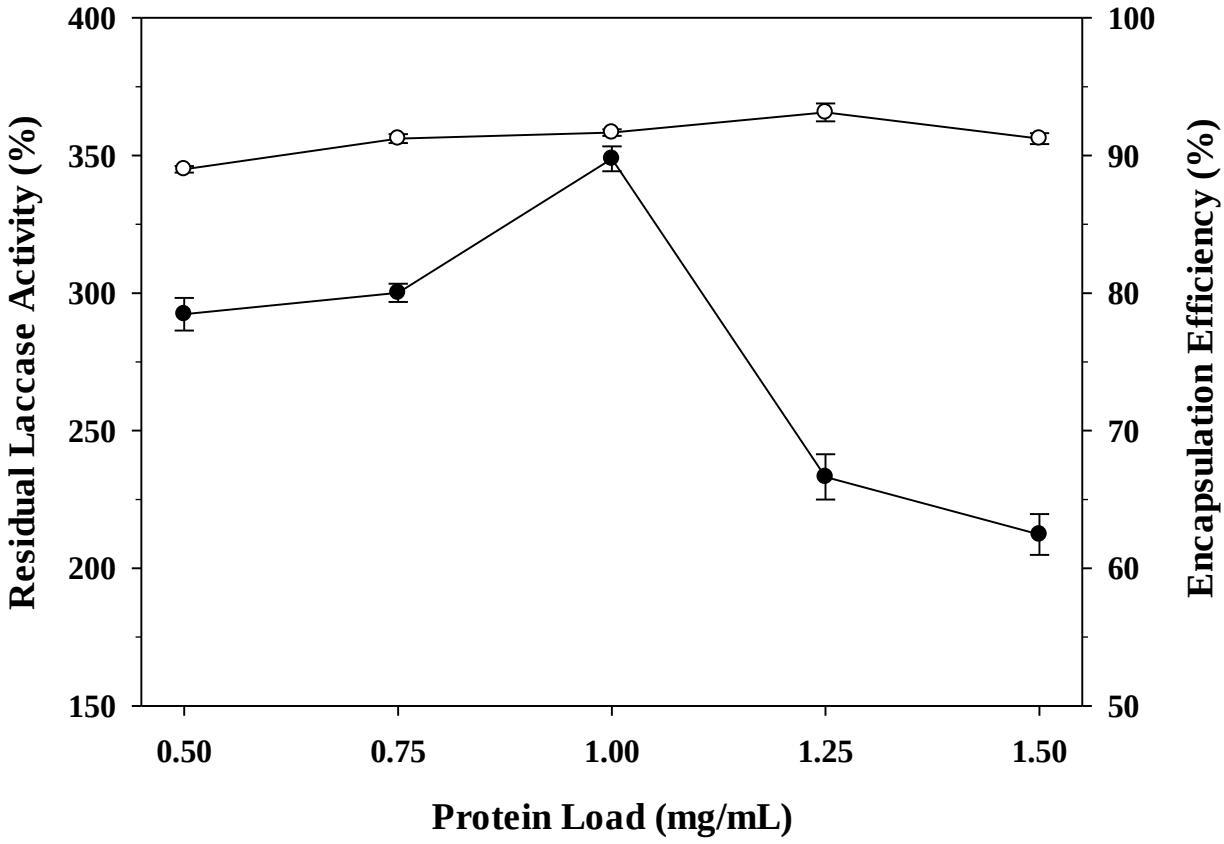


Figure 4.5. Effect of protein load (mg/mL sol) on the residual enzyme activity (●) and the encapsulation efficiency (○) of laccase, under standard conditions of water to silane ratio (2), 4 µmol HCl, 6 h of gel drying time and 24 h of gel aging time, with relative standard deviation of 0.2 to 8.0%.

4.4.5. Effects of protein load on the residual laccase activity and EE

The effect of protein loading in the sol-gel preparation on the residual laccase activity and the EE was investigated (Fig. 4.5). The results indicate an increase in the residual laccase activity with the increase in the protein load, where residual enzyme activities of 292, 300 and 349% were obtained, respectively, for 0.50, 0.75 and 1.00 mg protein/mL. Further increase in the protein concentration to 1.25 and 1.50 mg/mL resulted in a relatively lower residual laccase activity of 233 and 212%, respectively. The EE of the laccase extract, with protein loads of 0.50 to 1.50 mg/mL, ranged from 89 to 91%.

Similar findings by Rekuć *et al.* (2009) were reported for the immobilization of laccase, from *Cerrena unicolor*, on mesostructured siliceous cellular foams; a considerable decrease in both enzyme activity and EE was determined, with an increase in the protein load above 1 mg/mL. Lloret *et al.* (2011) reported a decrease, from 82 to 59%, in the enzyme activity of the sol-gel encapsulated laccase, with an increase in the protein loading, from 2.2 to 22.0 mg/mL, respectively; this decrease in the residual activity may have resulted from the diffusional limitations of the substrate entering the entrapment matrix. Lloret *et al.* (2011) also suggested that at higher protein concentrations, the aggregation of enzymes could lead to their improper distribution inside the encapsulation matrix and may ultimately result in a decrease in their catalytic activity.

4.5. Conclusion

The silica sol-gel encapsulation was found to be an effective method for the encapsulation of laccase enzymatic extract, with an enhanced residual enzyme activity and an EE of over 87%. The experimental findings demonstrated the effects of selected process conditions, involved in the silica sol-gel encapsulation of the laccase extract. The results showed that the residual laccase activity decreased concomitantly with an increase in the gel drying time; however, longer aging times resulted in a relatively higher residual enzyme activity. In addition, an increase in the *r*, HCl content and protein loading up to a certain limit, resulted in a comparatively higher laccase activity. Overall, the results showed the significance of the effect of each investigated parameter on the residual laccase activity and EE, which is of importance for the development of an optimized method for its encapsulation in silica sol-gel.

CONNECTING STATEMENT TO CHAPTER V

The encapsulation of laccase in silica sol-gel was found to be an efficient method for its immobilization and resulted in an enhanced residual enzyme activity (Chapter IV). Chapter V reports on the optimization for the sol-gel encapsulation of a laccase enzymatic extract, using response surface methodology (RSM). The encapsulated laccase was also characterized in terms of optimum pH, temperature stability, kinetic parameters, enzyme re-usability and storage stability.

CHAPTER V

OPTIMIZATION OF SILICA SOL-GEL ENCAPSULATION OF LACCASE EXTRACT USING RESPONSE SURFACE METHODOLOGY

5.1. Abstract

The research work was aimed at the development of an optimized method for the encapsulation of a laccase enzymatic extract, obtained from *Coriolus hirsutus*, in silica sol-gel matrix. A central composite rotatable design was used to evaluate the effects of water to silane molar ratio (*r*), acid (HCl) content and protein loading on the residual laccase activity. The statistical analysis indicated that *r* and acid content exerted the most significant effects on the residual enzyme activity. The optimal conditions for the encapsulation of the laccase extract in silica sol-gel were obtained with, *r* of 6.78, an acid content of 3.22 µmol and a protein loading of 0.86 mg/mL sol. The subsequent experiments, carried out under these conditions, confirmed the validation of the predicted model. The sol-gel encapsulated laccase extract was also evaluated for its optimum pH and temperature, kinetic parameters, re-usability and its storage stability. The experimental findings indicated an enhancement of the sol-gel encapsulated enzyme stability and an increase in its catalytic efficiency as compared to that of the free one, with a k_{cat} of 77.98 and 56.24 min^{-1}, respectively.

5.2. Introduction

Although there is tremendous increase of interest in the biotechnological applications of enzymes in a wide range of industrial processes (Hobbs and Thomas, 2007; El Agha *et al*., 2008; Osman *et al*., 2008), their effective use may be hindered by their high production costs, low yields and increased sensitivity to the surrounding conditions, such as pH, temperature and denaturing agents (Duran *et al*., 2002). However, the efficient immobilization of enzymes can overcome such limitations and may also improve some of their native properties (Fernández-Fernández *et al*., 2013).

The immobilization of biomolecules in inorganic silica sol-gels is a fairly well-established method and has been performed at a large-scale (Vila-Real *et al*., 2010). The silica sol-gels have been successfully used as an immobilization matrix for many enzymes, since they offer various

advantages including inertness and chemical stability, high porosity, optical transparency and ease of preparation. In addition, the sol-gel encapsulated enzymes have also been associated with an enhanced stability and re-usability (Qiu and Huang, 2010). Despite the many advantages offered by this entrapment matrix, the silica sol-gel is often associated with certain limitations, including volume shrinkage, brittleness and cracking, excessive alcohol production and enhanced mass-transfer limitations (Vila-Real *et al*., 2010; Mansor *et al*., 2016).

Laccases (EC 1.10.3.2) belong to the group of multi-copper blue oxidases and have attracted considerable attention due to their wide range of substrate specificity and their intrinsic stability; laccases catalyze the mono-electronic oxidation of various substrate molecules, including phenols, substituted polyphenols, alkenes and lignin-related molecules (Sanlier *et al*., 2013). Laccases have also been reported to demonstrate exceptional oxidizing ability for substrates with relatively higher redox potentials, in the presence of mediators (Rekuć *et al*., 2009).

This work is part of ongoing research work in our laboratory (Gill *et al*., 2018a,b) aimed at the development of an efficient process for the encapsulation and biocatalysis of a laccase enzymatic extract for its use in various biotechnological applications. The purpose of the current study was to optimize the silica sol-gel encapsulation of the laccase extract, from *Coriolus hirsutus*, using response surface methodology (RSM). The independent and mutual interaction effects of the water to silane molar ratio (*r*), HCl content and protein loading have been investigated to obtain the optimal residual laccase activity in the silica sol-gel matrix. In addition, the sol-gel encapsulated laccase extract was also characterized in terms of its optimum pH, temperature stability, kinetic parameters, re-usability and its storage stability.

5.3. Materials and Methods

5.3.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). FA and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Tetraethyl orthosilicate (TEOS) (98% purity) and all other chemicals of HPLC grade or higher were obtained from

Fischer Scientific (Fair Lawn, N.J.). Ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). All reagents and buffers were prepared using Milli Q plus (Millipore) water.

5.3.2. Production and recovery of laccase extract from Coriolus hirsutus

Laccase, from *Coriolus hirsutus* biomass, was extracted according to the protocol outlined by Taqi (2012). The enriched enzymatic extract obtained by ultrafiltration was then lyophilized in the presence of the additive mannitol (2.5% w/v) and stored at -80°C for further analysis.

5.3.3. Procedure for silica sol-gel encapsulation of the laccase enzymatic extract

Initial screening trials were performed to identify the parameters that have the most significant effects on the encapsulation efficiency (EE) and the residual laccase activity (Gill *et al*., 2018b). The encapsulation of laccase extract in silica sol-gel was carried out according to a modification of the method described by Mohidem and Mat (2012a,b). Prior to gelation, the silica precursor tetraethylorthosilicate (TEOS), deionized water and hydrochloric acid (HCl) were homogenized using the PowerGen 125 homogenizer (KIA; Wilmington, N.C.); the mixture was subsequently stirred until a clear silica sol was obtained. An aliquot of laccase extract suspension, prepared in potassium phosphate buffer (0.01 M, pH 6.0), was added to the silica sol suspension to obtain a final protein concentration between 0.5 to 1.25 mg/mL TEOS sol. The condensation of the silica sol was facilitated by an immediate addition of the potassium phosphate buffer (0.4 M, pH 7.0) with continuous agitation, until the onset of gelation (1 to 3 min). The resulting gel was aged for 24 h at 4ºC and later crushed, washed thoroughly and recovered by vacuum filtration using Whatman filter paper (No. 1). The gel containing the immobilized enzyme was then lyophilized for 6 h at -42°C and stored under refrigeration to maintain its catalytic activity. All washing solutions were collected for protein determination.

5.3.4. Protein determination

The protein content of the free enzyme extract and washing solutions was determined according to a modification to the Lowry method (Hartree, 1972), using bovine serum albumin (BSA) as a standard for the calibration curve.

5.3.5. Laccase activity assay

The enzyme activity assay for both free and sol-gel encapsulated laccase was carried out according to the method described by Bou-Mitri (2013). The enzyme activity was measured (50°C) by monitoring spectrophotometrically (Beckman Instruments Inc., San Raman, CA) the decrease in the absorbance at 320 nm as a result of the laccase catalyzed oxidation of FA. The specific activity was defined as µmol of converted ferulic acid/mg protein/min. All laccase assays were performed in triplicate trials and were run simultaneously with a blank reaction containing everything except the enzyme.

The residual laccase activity was defined as, the ratio of the specific activity of the encapsulated laccase to the free one, respectively, and was calculated as follows:

$$\text{Residual laccase activity (\%)} = (SA_{imm)} / (SA_{free)} \text{ x } (100) \text{ ……..... (I)}$$

Where, SA_{free} and SA_{imm} were the specific activities of the free and encapsulated laccase extract, respectively and were defined as µmol converted substrate (ferulic acid/mg/protein/min).

5.3.6. Experimental design for response surface methodology (RSM)

A 'five-level-by-three-factor' central composite rotatable design (CCRD) was employed to optimize the encapsulation of laccase extract in silica sol-gel matrix. The three independent variables used in this study with their corresponding levels were, X_1 as the water to silane molar ratio (r) (2.0 to 8.0), X_2 as the HCl content (2.0 to 8.0 µmol) and X_3 as the protein loading (0.50 to 1.25 mg/mL TEOS sol). The full factorial design was constructed from 16 factorial points (levels ± 1), 12 axial points (levels ± α) and 6 replicates in central point, resulting in a total of 34 experimental runs. All experiments were conducted in a randomized order, with duplicate measurements of the residual laccase activity for each run. Table 5.1 shows the variables with their coded and uncoded values. The residual laccase activity (%) was quantified as the response Y.

5.3.7. Statistical analysis

For predicting the response Y, the experimental data obtained was fitted to a quadratic polynomial regression model, using the Design Expert software (Version 8.0, Stat-Ease, Inc., Minneapolis, MN):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad \text{..... (II)}$$

where, Y is the dependent variable (residual laccase activity) to be modelled, while X_i and X_j are the independent variables. The regression coefficients of the model are represented by β_0 (constant term), β_i (linear coefficient), β_{ii} (quadratic coefficient) and β_{ij} (interaction coefficient). The analysis of variance (ANOVA) of the model was investigated to determine its adequacy in describing the observed data, where the coefficient of determination (R^2) indicated the variability of the fit and was checked using the F-test. Contour plots were drawn using the fitted model, to illustrate the major interactive effects of the independent variables on the dependent one.

5.3.8. Characterization of sol-gel encapsulated laccase

5.3.8.1. Effect of pH on laccase activity

The effect of pH on the activity of free and encapsulated laccase enzymatic extract was determined by measuring the enzyme activity over a wide range of pH values of 2.5 to 7.0, using citrate phosphate buffer (0.1 M). All enzyme preparations were pre-incubated for 1 h, at their respective pH values, before carrying out the enzymatic assay according to the protocol described previously, but with use of the selected pH value (2.5 to 7.0).

5.3.8.2. Effect of reaction temperature on laccase activity

The effect of reaction temperature on the free and encapsulated laccase extract activity was investigated by their pre-incubation for 1 h, using a wide range of temperatures of 25 to 70°C. All the enzymatic assays were carried out according to the protocol described previously, but with use of the selected reaction temperature (25 to 70°C).

5.3.8.3. Determination of kinetic parameters of the encapsulated laccase extract

The kinetic parameters of the free and sol-gel encapsulated laccase extract were determined by measuring the laccase activity within a range of FA concentrations (0 to 50 mM). The

Michaelis-Menten constants, K_m and V_{max}, were determined using the Lineweaver-Burk plots and the turnover number, k_{cat} was calculated from the ratio of V_{max}/K_m. All activity assays were performed by means of triplicate trials.

5.3.8.4. Re-usability of the encapsulated laccase extract

The re-usability of the sol-gel encapsulated laccase extract was investigated by measuring the enzyme activity, for six continuous reaction cycles. The specific activity of the enzyme in the first cycle was expressed as 100% and the relative residual laccase activity was defined as the percentage specific activity obtained in a defined cycle, as compared to that in the first one.

5.3.8.5. Storage stability of the encapsulated laccase extract

The storage stability of the free and encapsulated laccase extracts was determined by their storage at temperatures of 25 and 4°C, for a period of 6 weeks. The relative residual laccase activity was defined as, the percentage specific activity of laccase at a selected storage time, to that of the initial one.

5.4. Results and Discussion

5.4.1. Optimization of the encapsulation of laccase extract in silica sol-gel matrix

5.4.1.1. Model fitting and ANOVA

Response surface methodology (RSM) was used to optimize the encapsulation of laccase extract in silica sol-gel matrix and to assess the individual and interactive effects of the selected parameters, including *r*, HCl content and protein loading, on the residual laccase activity. The experimental design was carried out based on a central composite rotatable design (CCRD). The actual and coded values of the experimental data are presented in Tables 5.1 and 5.2. The multiple regression analysis was applied, using the Design Expert Version 8.0 software, where the best fitting model was determined. The adequacy and the significance of the model were confirmed with the ANOVA (Table 5.3). The results indicated that the encapsulation of laccase extract in sol-gel was most significantly described by the following quadratic polynomial model:

$$\text{Residual laccase activity (\%)}\ (Y) = +335.91 + 36.74X_1 - 23.91X_2 + 23.86X_3 - 51.03X_1X_2 - 3.10X_1X_3 + 23.00X_2X_3 + 16.51X_1^2 - 18.93X_2^2 - 13.65X_3^2 \quad \text{(III)}$$

where, X_1 is the *r* value, X_2 is the HCl content and X_3 is the protein loading.

Table 5.1. Process variables and their levels used in the central composite rotatable design (CCRD).

		Coded levels				
Variable	Name	-1.682	-1	0	+1	+1.682
X_1	Water:Silane molar ratio (r)	2.00	3.22	5.00	6.78	8.00
X_2	HCl Content (μmol)	2.00	3.22	5.00	6.78	8.00
X_3	Protein Loading (mg/mL silica sol)	0.50	0.65	0.88	1.10	1.25

Table 5.2. Experimental design for a 5-level-3-factor central composite rotatable design.

Run no.	Water:Silane (r)[a] X_1	HCl content (µmol)[b] X_2	Protein loading (mg/mL)[c] X_3	Residual specific activity (%)[d] Y
1	3.22[e] (-1.000)[f]	3.22[e] (-1.000)[f]	1.10[e] (1.000)[f]	267.01
2	8.00 (1.682)	5.00 (0.000)	0.88 (0.000)	468.94
3	6.78 (1.000)	6.78 (1.000)	0.65 (-1.000)	246.22
4	5.00 (0.000)	5.00 (0.000)	0.50 (-1.682)	233.82
5	5.00 (0.000)	5.00 (0.000)	1.25 (1.682)	304.94
6	3.22 (-1.000)	3.22 (-1.000)	0.65 (-1.000)	268.25
7	2.00 (-1.682)	5.00 (0.000)	0.88 (0.000)	305.24
8	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	332.32
9	3.22 (-1.000)	6.78 (1.000)	0.65 (-1.000)	237.43
10	6.78 (1.000)	6.78 (1.000)	1.10 (1.000)	338.81
11	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	351.88
12	3.22 (-1.000)	6.78 (1.000)	0.65 (-1.000)	307.03
13	6.78 (1.000)	3.22 (-1.000)	1.10 (1.000)	413.54
14	5.00 (0.000)	8.00 (1.682)	0.88 (0.000)	234.99
15	2.00 (-1.682)	5.00 (0.000)	0.88 (0.000)	297.61
16	8.00 (1.682)	5.00 (0.000)	0.88 (0.000)	422.79
17	5.00 (0.000)	2.00 (-1.682)	0.88 (0.000)	314.33
18	5.00 (0.000)	5.00 (0.000)	1.25 (1.682)	353.31
19	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	321.87
20	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	323.68
21	6.78 (1.000)	3.22 (-1.000)	0.65 (-1.000)	457.06
22	3.22 (-1.000)	3.22 (-1.000)	0.65 (-1.000)	255.81
23	5.00 (0.000)	8.00 (1.682)	0.88 (0.000)	248.90
24	5.00 (0.000)	2.00 (-1.682)	0.88 (0.000)	295.48
25	3.22 (-1.000)	6.78 (1.000)	1.10 (1.000)	352.54
26	5.00 (0.000)	5.00 (0.000)	0.50 (-1.682)	261.30
27	6.78 (1.000)	3.22 (-1.000)	1.10 (1.000)	446.40
28	6.78 (1.000)	6.78 (1.000)	0.65 (-1.000)	217.39
29	3.22 (-1.000)	6.78 (1.000)	1.10 (1.000)	372.03
30	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	332.22
31	6.78 (1.000)	6.78 (1.000)	1.10 (1.000)	317.27
32	6.78 (1.000)	3.22 (-1.000)	0.65 (-1.000)	431.56
33	3.22 (-1.000)	3.22 (-1.000)	1.10 (1.000)	290.38
34	5.00 (0.000)	5.00 (0.000)	0.88 (0.000)	359.65

[a] Water:Silane (r) was the molar ratio of water and silica precursor, tetraethylorthosilicate (TEOS) in the pre-gel sol.
[b] HCl content in µmol was the concentration of the acid in the pre-gel silica sol.
[c] Protein Loading was the concentration of protein in the laccase extract in mg/mL of the silica sol, introduced for encapsulation.
[d] Residual Specific Activity (%) was the percentage specific activity of the encapsulated laccase to that of the free one, multiplied by 100.
[e] Experimental values.
[f] Coded values of the experimental values.

Table 5.3. The analysis of variance (ANOVA) and model coefficients for the response surface quadratic model.[a,b]

Source	Sum of squares	df[c]	Mean square	*F*-value	*p*-value[d]
Model	1.429E+005	9	15880.28	32.31	< 0.0001
X_1 (Water:Silane)	36876.19	1	36876.19	75.02	< 0.0001
X_2 (HCl content)	15614.47	1	15614.47	31.76	< 0.0001
X_3 (Protein loading)	15543.76	1	15543.76	31.62	< 0.0001
X_1X_2	41661.91	1	41661.91	84.75	< 0.0001
X_1X_3	154.07	1	154.07	0.31	0.5808
X_2X_3	8460.78	1	8460.78	17.21	0.0004
X_1^2	6143.02	1	6143.02	12.50	0.0017
X_2^2	8076.88	1	8076.88	16.43	0.0005
X_3^2	4202.67	1	4202.67	8.55	0.0074
Residual	11797.52	24	491.56		
Lack of Fit	3221.14	5	644.23	1.43	0.2596
Pure Error	8576.38	19	451.39		
Cor Total	1.547E+005	33			

[a] $R^2 = 0.9237$

[b] Coefficient of variation = 6.86%

[c] Degree of freedom (df)

[d] *p*-value ≤ 0.05 indicates statistical significance.

The quadratic model was statistically significant, with an *F*-value of 32.31, a *p*-value of < 0.0001 and a coefficient of determination (R^2) of 0.924, respectively. The lack of fit F-value of 1.43 was non-significant (*p*-value >0.05). These experimental results imply that the model adequately represents the relationship between the residual enzyme activity (%) of the encapsulated laccase with the individual parameters, within the selected experimental ranges.

The *F*- and *p*-values can also be used to determine the significance and interactions of the independent variables (Seo *et al*., 2012). The results presented in Table 5.3 indicate that the residual laccase activity was most significantly affected by X_1-water to silane molar ratio (*F*-value of 75.02, *p*-value <0.0001), which was followed by X_2-HCl content (*F*-value of 31.76, *p*-value <0.0001) and X_3-protein loading (*F*-value of 31.62, *p*-value <0.0001). In addition, other than X_1X_3, all the interactive and quadratic terms were significant (*P*-values ≤0.05); the water to silane molar ratio (X_1) and HCl content (X_2) had the most important interaction effects (X_1X_2; F-value of 84.75) and the HCl content (X_2^2; F-value of 16.43) was the most important quadratic term affecting the residual laccase activity. Moreover, the model (Equation III) shows antagonistic interactive effects, where a positive sign represents a synergistic interaction, while a negative sign indicates an antagonistic one (Sorour, 2010).

5.4.1.2. Effects of variables on the residual laccase activity

The relationships between a given individual parameter and the residual laccase activity (%) can be better understood from the 2-D contour plots generated from the predicted model. The contour graphs (Fig. 5.1a-c) show the effects of *r* (X_1) and HCl content (X_2), at three different protein loadings (X_3; 0.65, 0.88, 1.10 mg/mL silica sol) as well as their mutual interaction effects on the residual laccase activity. All three graphs (Fig. 5.1a-c) display similar trends, where the residual laccase activity increased with a concomitant increase in *r* and a decrease in the acid content. The diagonal ellipses in the contour graphs (Fig. 5.1a-c) indicate the opposite interactive effect between *r* and the acid content. Furthermore, an increase in the protein loading resulted in a relatively higher residual laccase activity; however, the maximum was achieved at a concentration of 0.88 mg protein/mL sol. Beyond this point, a further increase in the protein loading resulted in a decrease in the residual enzyme activity, which may be due to diffusional and mass transfer limitations caused by the enzyme aggregation within the support matrix (Bou-Mitri, 2013; Zhang *et al*., 2013).

In addition, the experimental results also indicated that at higher protein loadings of 0.88 and 1.10 mg/mL, the residual laccase activity increased even with higher acid contents.

Using RSM, Mansor *et al*. (2016) optimized the synthesis conditions for the laccase encapsulation in silica microparticles; these authors reported a significant increase in the laccase specific activity with a concomitant increase in the *r* value and suggested that the interactive effect between *r* and the acid concentration resulted in a "bulls-eye" pattern contour plot. Yang *et al*. (2009) reported the sol-gel immobilization of lipase from *Arthobacter sp.*, and suggested that the decrease in the residual enzyme activity with lower values of *r* may be attributed to enzyme agglomeration as well as to an increase in alcohol condensation, which could result in enzyme denaturation. Dickson *et al.* (2009) indicated that the *r* value influenced the silica sol hydrolysis and condensation reactions, which may have affected the final gel structure; an increase in the water content favored a complete hydrolysis and resulted in a more stable sol.

Figures 5.1d-f show the interactive effects of HCl content (X_2) and protein loading (X_3), at different values of *r* (X_1; 3.22, 5.00, 6.78). At a lower *r* of 3.22, the residual laccase activity increased simultaneously with the increase in protein loading and acid content; in contrast, at *r* values of 5.00 and 6.78, a higher residual enzyme activity was obtained with the decrease in HCl content. These results indicate that a relatively higher residual laccase activity could be obtained even with an increase in acid content, but with a simultaneous increase in protein loading. Aziz *et al*. (2012) reported that with a higher enzyme concentration, the probability of the enzyme-substrate collision may increase and hence could result in a relatively enhanced enzyme activity. Overall, the experimental findings (Fig. 5.1) indicated that maximum response was achieved with a combination of high *r* and low acid content.

Curran and Steigman (1999) investigated the effect of acid content on the silica sol-gel morphology and reported that the increase in acid content resulted in a concomitant increase in gel porosity and in surface area as well as a decrease in the gelation time. Magner (2013) reported that porous supports, with high surface areas, were ideal matrices for enzyme immobilization, where

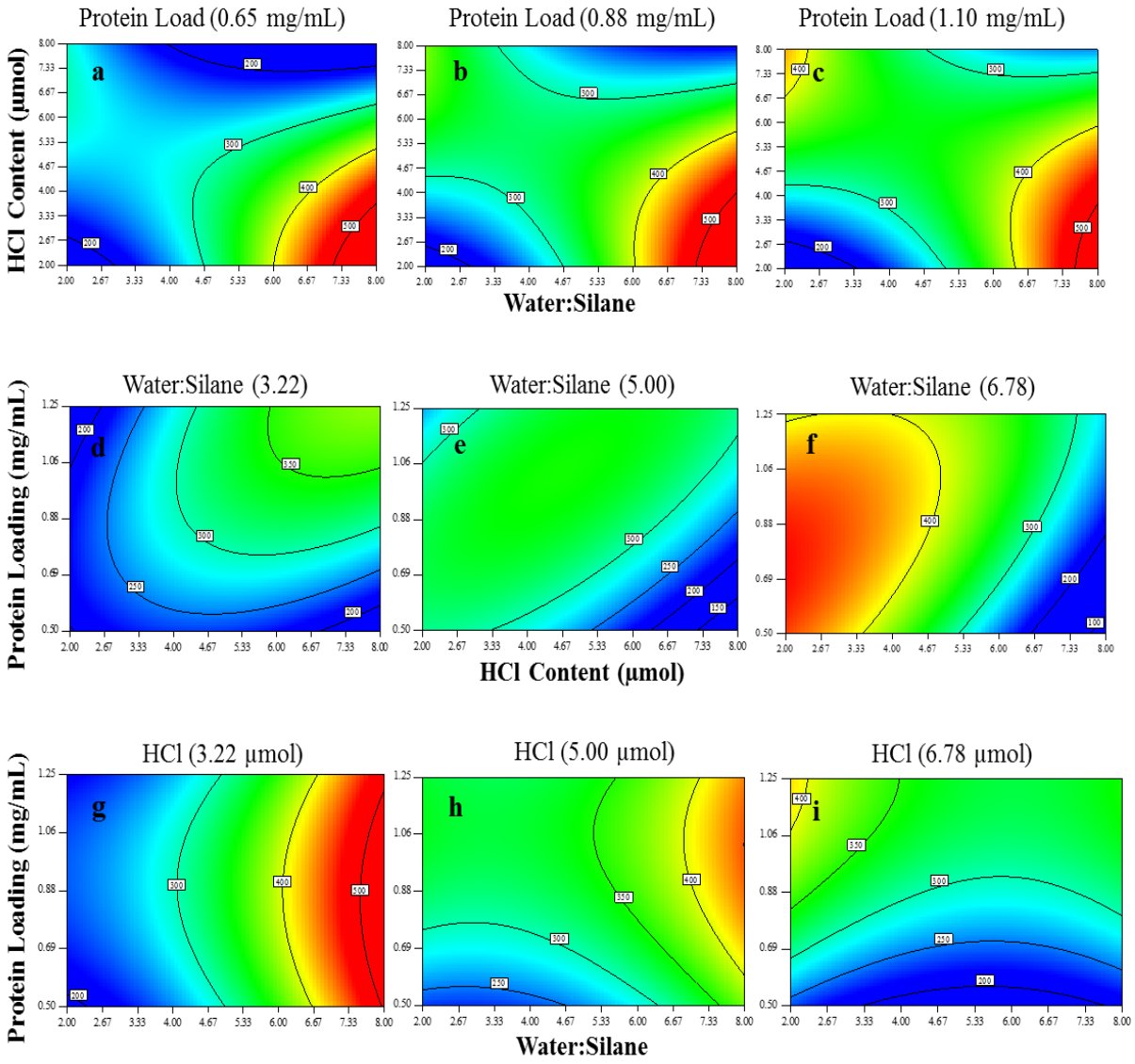


Figure 5.1. Contour plots of predictive models of the residual enzyme activity (%) of the encapsulated laccase extract at protein loadings of 0.65, 0.88 and 1.10 mg/mL (a-c), water to silane molar ratio (r) of 3.22, 5.00, 6.78 (d-f) and HCl content of 3.22, 5.00 and 6.78 µmol (g-i). The predicted responses are indicated by the numbers inside the contour plots.

the rate of the reaction was directly proportional to their respective surface areas; this author suggested that such supports did not have mass transfer limitations, hence, promoted product diffusion with enhanced substrate accessibility. Overall, the experimental findings (Fig. 5.1d-f) indicate that the higher residual laccase activity, obtained with an increased HCl content and protein loading, may be due to an increase in gel porosity and to a reduced gelation time; the use of shorter gelation times prevent the long-term exposure of the entrapped enzyme to the surrounding ethanol, which could lead to a relatively lower residual activity (Dickson and Ely, 2011). Although a combination of high acid content and protein loading can be used to increase the residual laccase activity, such gels are generally associated with problems of excessive shrinkage and cracking (Rao and Bhagat, 2004). In addition, the results (Fig. 5.1d-f) suggest that the increased effect of the X_2 on the response (Y) can only be obtained at higher concentrations of the enzyme, which could hence limit its economic viability.

Figures 5.1g-i illustrate the interaction between the r (X_1) and protein loading (X_3), at different HCl contents (X_2; 3.22, 5.00, 6.78 µmol). These results indicate that at a low acid content of 3.22 µmol, the residual laccase activity increased with the increase in r and protein loading, up to 6.78 and 0.88 mg/mL, respectively; however, a further increase in acid content resulted in a decrease in the enzyme activity. In addition, at higher HCl contents of 5.00 and 6.78 µmol, the residual laccase activity increased only with lower r values and higher ones of the protein loading. The increase in the residual laccase activity, obtained at high acid content and low r, could be a result of an increase in gel porosity and surface area (Huang *et al*., 1999; Rao and Bhagat, 2004).

5.4.1.3. Optimal conditions and model validation

The optimal conditions found for the silica sol gel encapsulation of laccase extract were r of 6.78, HCl content of 3.22 µmol and protein loading of 0.86 mg/mL sol. Under these conditions, the predicted value for the relative residual laccase activity was 445.27%. In order to validate the proposed model, three replicate trials were carried out under the optimal conditions; the experimental data showed that the mean of the residual laccase activity of the encapsulated enzyme was 443.31% ± 5.52% higher than that for the free one, which indicates a valid correlation between

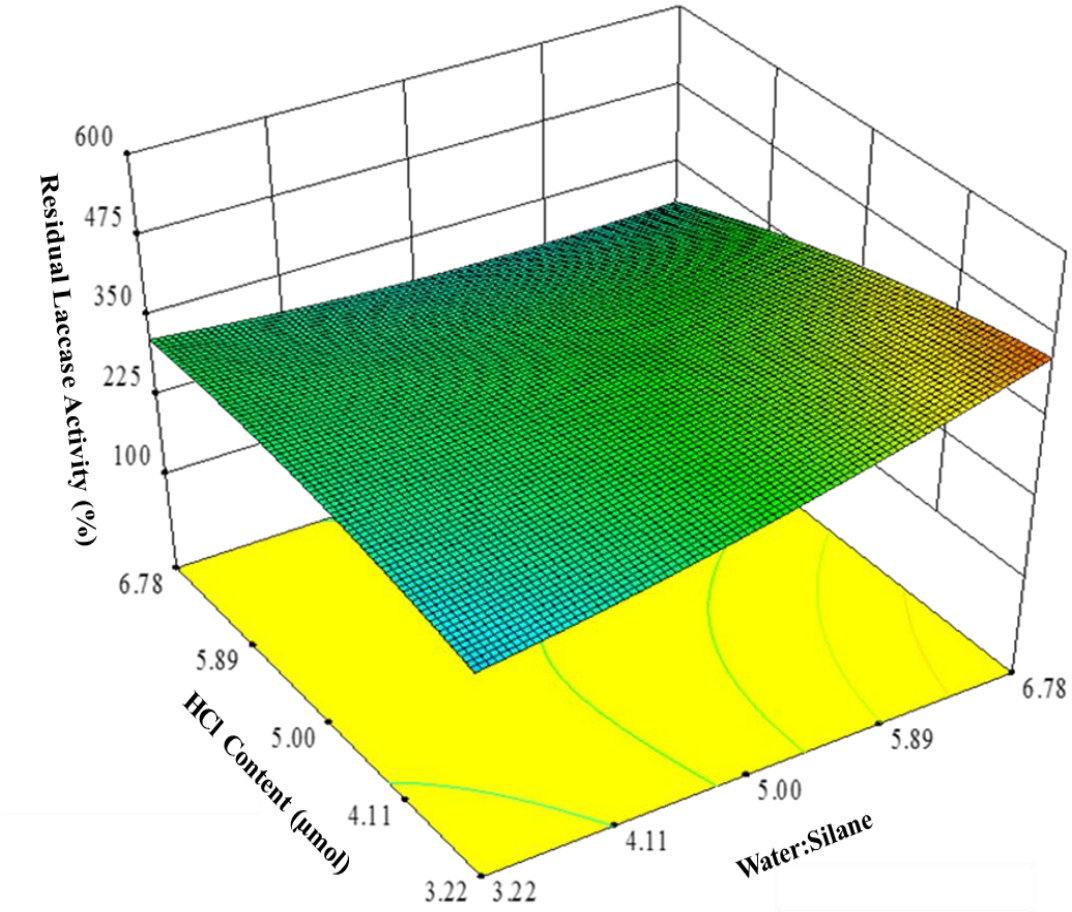


Figure 5.2. Response surface plot showing the effect of water to silane molar ratio (*r*) and HCl content, and their mutual interaction on the residual laccase activity. The protein loading is constant at 0.86 mg protein/mL sol.

the experimental results and the statistical model's predictions. The results presented in Figure 5.2 confirm the validation of the RSM model and the three-dimensional plot generated from the fitted model equation.

Similarly, Mansor *et al*. (2016) investigated the parameters for the encapsulation of laccase in silica microparticles, using the Box-Behnken design; the conditions for the optimum specific catalytic activity were, *r* of 5.44, HCl concentration of 2.52 µM, and protein loading of 3.83 mg/mL, with corresponding predicted and determined a specific activity of 301.7 U/g and 298.4 U/g, at a coefficient of determination R^2 of 0.89.

5.4.2. Characterization of free and sol-gel encapsulated laccase

5.4.2.1. Effect of pH on laccase activity

Using FA as a substrate, Figure 5.3 demonstrates the effect of a wide range of pH (2.5 to 7.0) on the specific activity of the sol-gel encapsulated laccase extract. The results indicate that although the optimum pH for both free and encapsulated laccase extracts was 5.0, the sol-gel encapsulated enzyme exhibited higher relative specific activity. The results also show that both free and encapsulated laccase extracts retained a better relative enzyme activity at pH values of 2.5 to 5.5.

Similarly, Bou-Mitri (2013) reported that the optimum pH for laccase extract in ternary micellar system media, using syringaldazine as a substrate, was 5.0. Although Shamsuri *et al*. (2012) reported an optimum pH value of 5.0 and 6.0, respectively for the free and sol-gel encapsulated laccase extracts, the optimal pH for the encapsulated enzyme shifted slightly towards the acidic region as compared to that for the free one. Mohidem and Mat (2009) also indicated an optimum pH of 6.0 and 5.0, respectively for the free and sol-gel encapsulated laccases.

5.4.2.2. Effect of temperature on laccase activity

The experimental findings shown in Figure 5.4 indicate that the relative activity of the sol-gel encapsulated laccase extract increased with the increase in temperature, up to 50°C; however, beyond this temperature, the encapsulated enzyme exhibited a higher relative activity as compared to that for the free one.

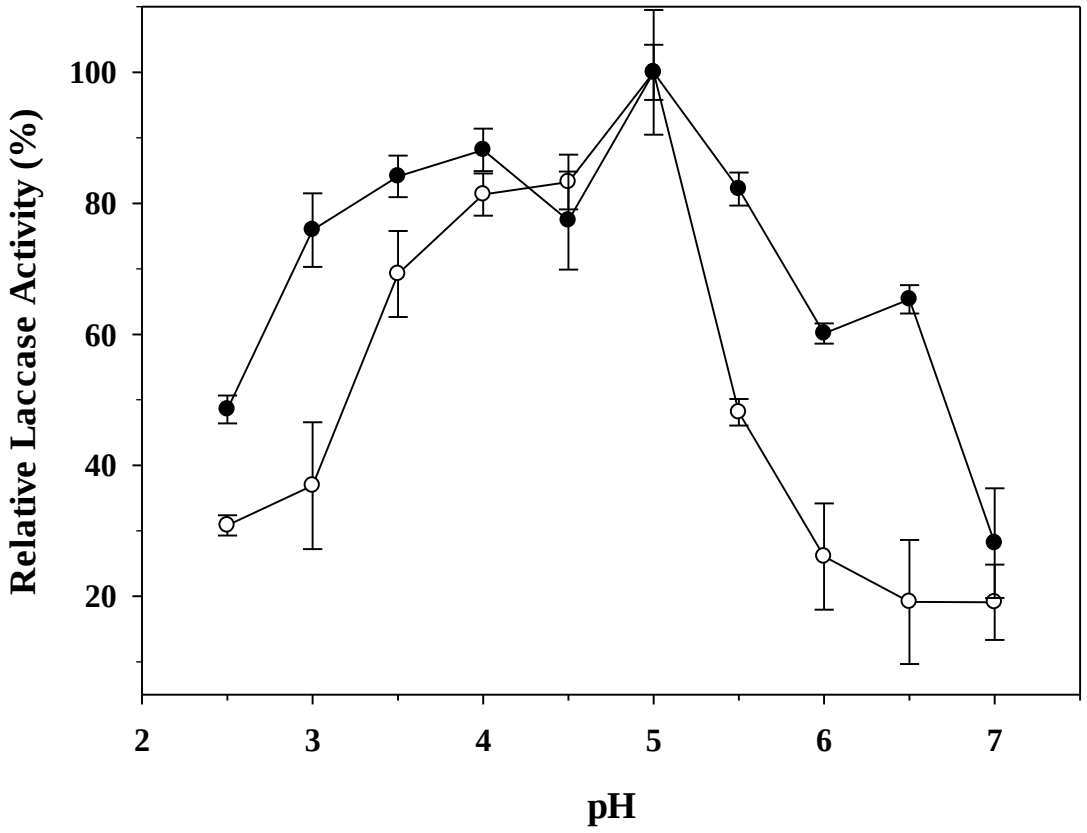


Figure 5.3. Effect of buffer pH on the specific activity of free (○) and sol-gel encapsulated (●) laccase, using ferulic acid as a substrate, with a relative standard deviation of triplicates between 1 and 10%. The relative laccase activity (%) was defined as the specific activity of laccase at a specific pH to that at the optimum pH.

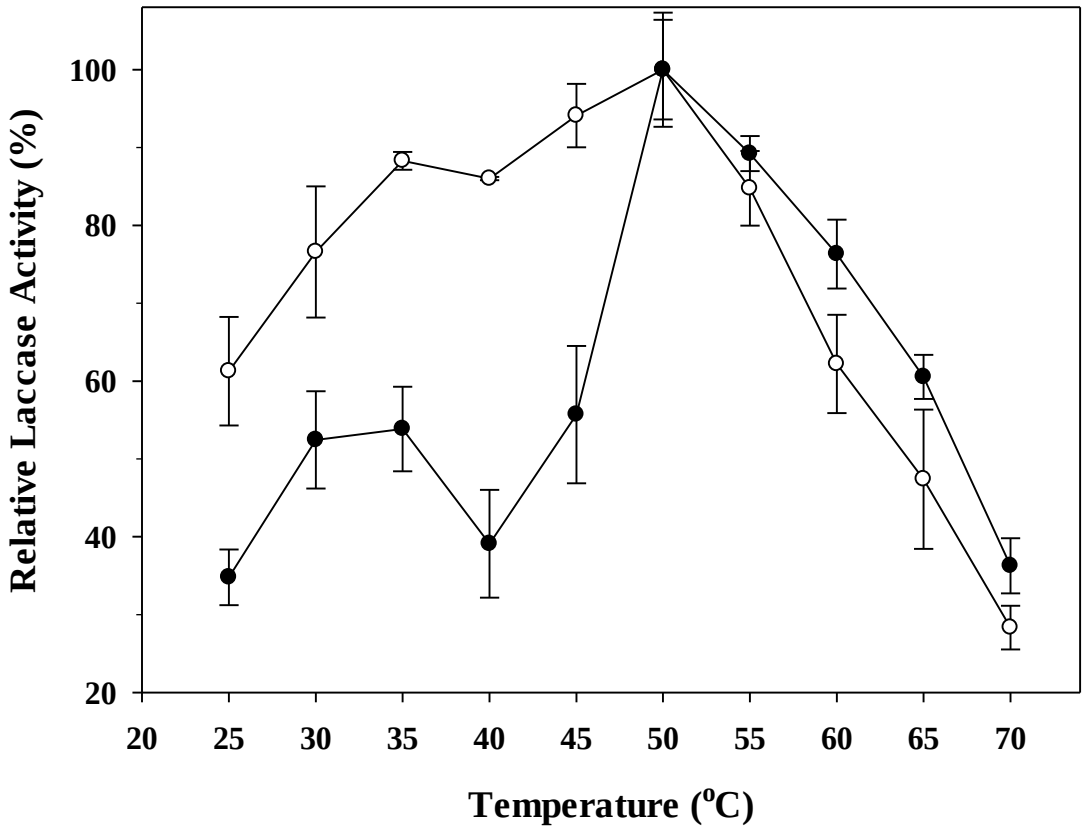


Figure 5.4. Effect of reaction temperature on the specific activity of free (○) and sol-gel encapsulated (●) laccase, assayed at an optimum pH of 5.0 with ferulic acid as a substrate. The relative laccase activity was defined as the specific activity of laccase at a certain temperature to that obtained at the optimum temperature. All trials were conducted in triplicates, with the relative standard deviations between 1 and 9%.

Similar findings by Mohidem and Mat (2012a) indicated that the sol-gel encapsulated laccase extract exhibited enhanced enzyme stability, at elevated temperatures, as compared to that for the free one; these authors suggested that the difference in the optimal temperature range could be due to the stiffening of the enzyme's tertiary structure, as a result of the encapsulation. Mohidem and Mat (2012a) also indicated that the increase in enzyme activity, up to a certain temperature, and its subsequent decrease beyond that temperature may be due to the enzyme aggregation. Similar findings by Shamsuri *et al*. (2012) indicated an increase in the laccase-catalyzed dye decolourization with the increase in temperatures, up to 40°C. Lloret *et al*. (2011) also reported that the sol-gel encapsulated laccase extract exhibited a broader stability profile, with a 10 to 20% higher relative activity than that for the free one.

5.4.2.3. Kinetic parameters of the sol-gel encapsulated laccase extract

The overall experimental findings for the sol-gel encapsulated laccase extract confirmed the Michaelis-Menten kinetics model (R^2 = 0.99). The results presented in Table 5.4 demonstrated a relatively higher K_m of 2.87 µM and V_{max} of 490.97 µmol/mg/min, as compared to that for the free one, with a K_m of 0.73 µM; V_{max} of 43.69 µmol/mg/min, respectively. The higher K_m value for the sol-gel encapsulated laccase indicates a reduced substrate affinity, which could be a result of the diffusional limitations, occurring within the matrix (Rekuć *et al*., 2009).

Mansor *et al*. (2016) investigated the kinetic parameters for the sol-gel encapsulated laccase extract, using ABTS as a substrate, and reported a reduced enzyme substrate affinity for the encapsulated enzyme which may be due to mass transfer limitations. In addition, Mansor *et al.* (2016) suggested that the lower k_{cat}/K_m value for the encapsulated laccase could be due to the substrate diffusion limitations and to the reduced protein flexibility upon immobilization. Using ABTS and DMP as a substrate, for the immobilization of laccase, Lettera *et al*. (2016) indicated a relatively higher K_m for DMP and a lower one for ABTS; these authors suggested that certain phenomena, such as electrostatic partitioning effects, substrate mass transfer limitations and enzyme conformational changes which occurred in the immobilized enzymes, may have affected their substrate-dependent properties. Likewise, Vila-Real *et al.* (2011) indicated relatively higher K_m values for the sol-gel encapsulated α-rhamnosidase and β-glucosidase as compared to their free counterparts and suggested that the mass-transfer limitations in the immobilized enzymes may have resulted in lower k_{cat} and $k_{cat}\ K_m^{-1}$ values.

Table 5.4. Michaelis-Menten kinetic parameters for the silica sol-gel encapsulated laccase extract.

Laccase extract	K_m [a]	V_{max} [b]	k_{cat} [c]	k_{cat}/K_m [d]
Free	0.73 (4.93) [e]	43.69 (6.70)	56.24 (1.77)	77.10 (3.17)
Sol-gel encapsulated	2.87 (3.80)	490.97 (8.84)	76.98 (5.05)	26.85 (1.26)

[a]The Michaelis-Menten constant (K_m), was expressed in µM.

[b]The apparent maximum velocity (V_{max}) of laccase was defined as the µmol converted substrate/mg encapsulated or free protein/min.

[c]Catalytic efficiency (k_{cat}) was defined as the ratio of K_m/V_{max}.

[d]The specificity constant was expressed as the ratio of k_{cat}/K_m.

[e]Relative standard deviation (%) was calculated as the standard deviation divided by the mean of triplicate trials, multiplied by 100.

5.4.2.4. Re-usability of the encapsulated laccase extract

The re-usability profile of the silica sol-gel encapsulated laccase extract is shown in Figure 5.5. The experimental findings indicated that the encapsulation of the enzyme resulted in a relative stability of its activity during 5 continuous reaction cycles, with 76% of the relative enzyme activity retained in the 6^{th} cycle.

Mansor *et al*. (2016) investigated the recycling stability of the sol-gel encapsulated laccase extract and indicated that the encapsulated enzyme retained 90% of its initial catalytic activity, after 10 cycles; these authors also conducted a leaching study and suggested that the subsequent decrease in the enzyme activity was not due to enzyme leakage, but rather to a denaturation of the enzyme. Similarly, Qiu and Huang (2010) reported that 80.2% of laccase activity was retained in the sol-gel immobilized enzyme in continuous flow reactors, after 120 h.

5.4.2.5. Storage stability of the encapsulated laccase extract

The storage stability of the free and sol-gel encapsulated laccase extracts was investigated at 25°C and 4°C, for a period of 6 weeks. The results presented in Figure 5.6 indicate that the encapsulated enzyme retained up to 99 and 86% of its initial activity, at 4 and 25°C, respectively, whereas it was 56 and 5%, respectively for the free one; in addition, at 25°C the free laccase extract lost most (86%) of its initial enzyme activity after the first week of its storage.

Mohidem and Mat (2012a) indicated that the encapsulated laccase extract retained its activity after 70 days of storage at 27°C, whereas the free one completely lost its activity by the end of the same period. Li *et al*. (2011) reported that the silica glass immobilized horseradish peroxidase extract retained 85% of its activity after 4 weeks of storage, whereas the free one lost 90% of its activity for the same period. Similarly, Qiu and Huang (2010) reported that the sol-gel immobilized laccase extract retained 72.1% of its initial activity after 6 weeks storage at 25°C, whereas that for the free one was only 15.6%.

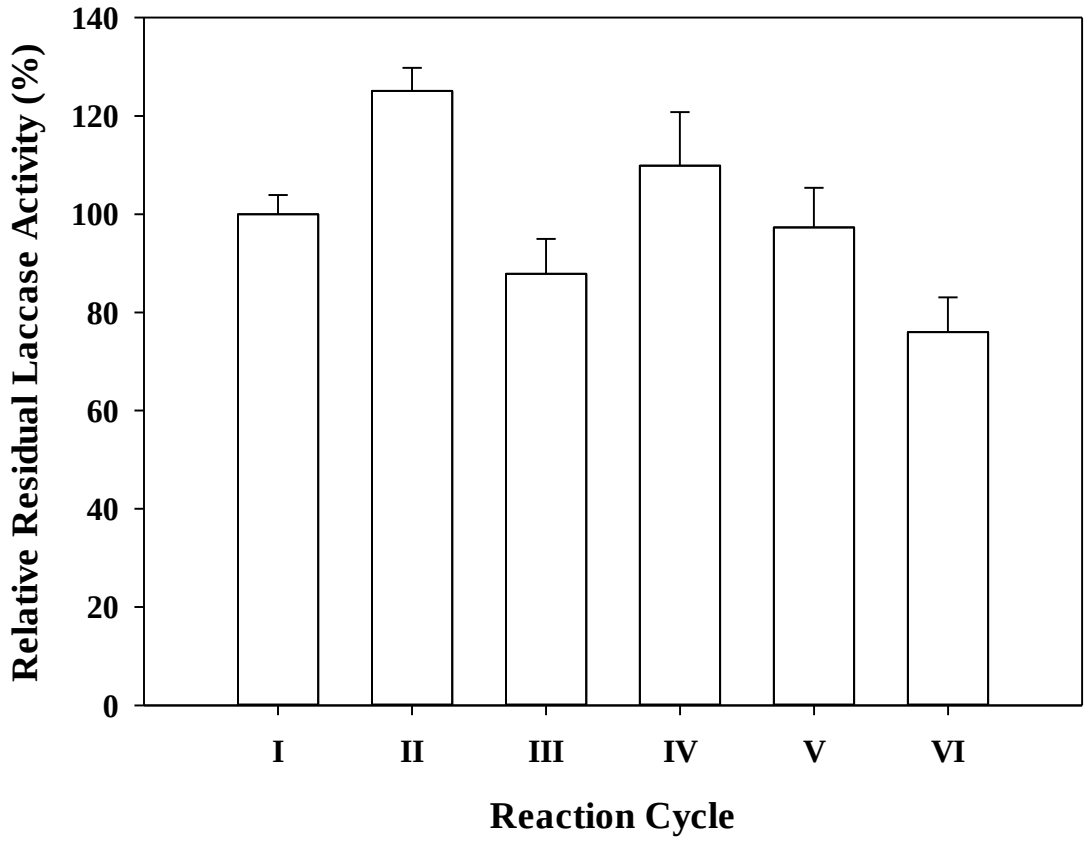


Figure 5.5. Re-usability of laccase extract encapsulated in silica sol-gel matrix. The relative residual laccase activity (%) was defined as its specific activity obtained at a particular cycle to its initial specific activity at time 0. The results are means of triplicate trials with relative standard deviations between 2 and 11%.

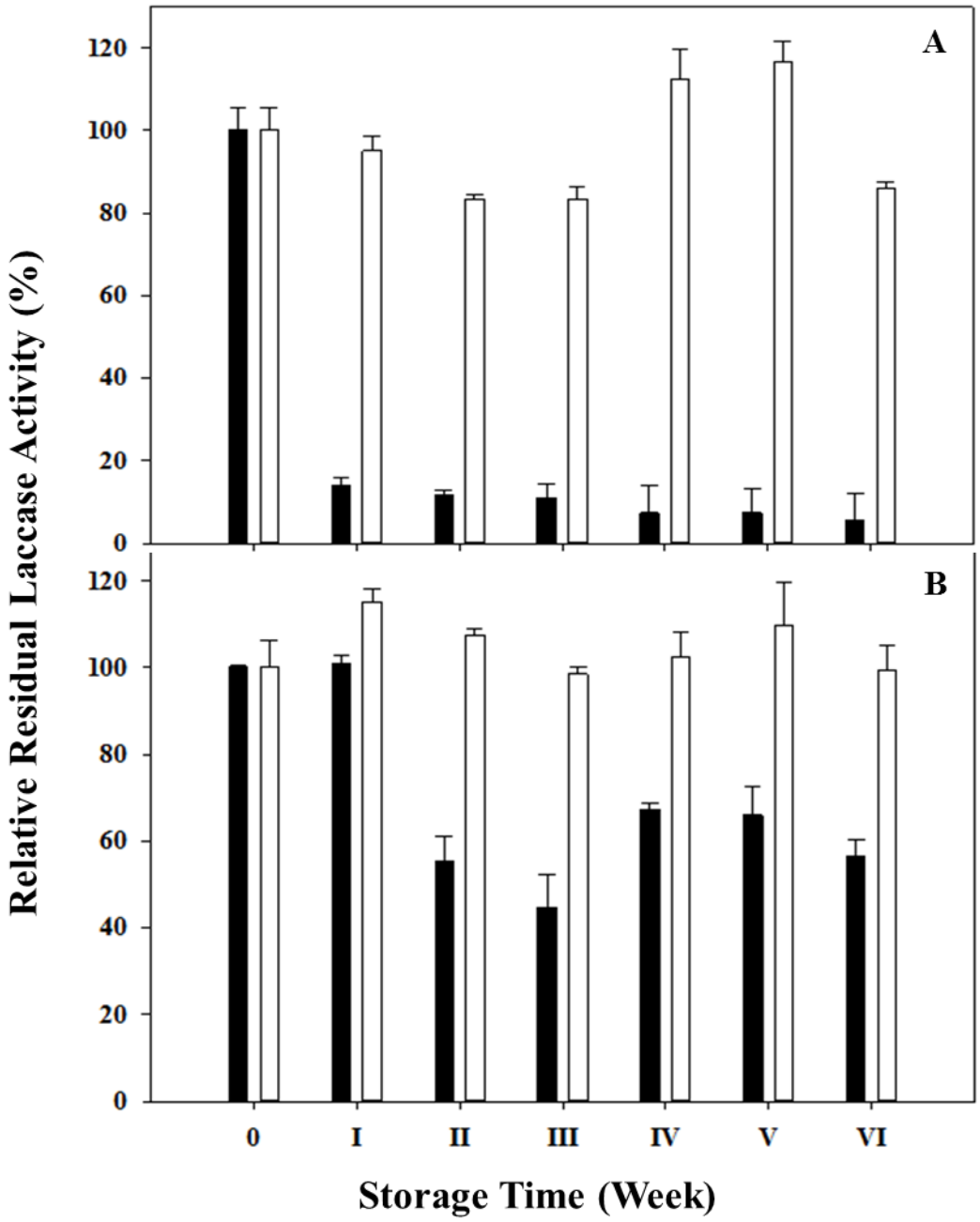


Figure 5.6. Effect of storage temperatures, 25°C (A) and 4°C (B) on the stability of free (■) and silica sol-gel encapsulated laccase (□). The relative residual laccase activity was defined as the specific activity of the free or the encapsulated laccase extract after storage at a certain storage time, to that at week 0. All results were determined by means of triplicate trials with the relative standard deviations between 1 and 10%.

5.5. Conclusion

An efficient method was developed for the encapsulation of a laccase enzymatic extract in silica sol-gel matrix. The optimization of the method was successfully performed, using response surface methodology with the central composite rotatable design (CCRD). Among the investigated variables, the water to silane molar ratio (*r*) was the most significant independent variable that affected the relative residual laccase activity; in addition to this, the *r* and acid content showed the most significant interaction effects, with their opposite effects. Overall, the sol-gel encapsulation of laccase extract resulted in a more stable enzyme.

CONNECTING STATEMENT TO CHAPTER VI

Using the central composite rotatable design (CCRD), an optimized method for the silica sol-gel encapsulation of laccase enzymatic extract was developed as described in Chapter V. Chapter VI covers the biocatalysis of the sol-gel encapsulated laccase enzymatic extract in solvent-free media (SFM). Selected parameters for the biocatalysis of laccase in SFM, including the enzymatic protein content, reaction temperature and agitation speed, were investigated with the use of FA as the substrate model. The kinetic parameters of the enzymatic laccase extract in SFM were also determined, using selected phenolic substrate models.

CHAPTER VI

BIOCATALYSIS OF FREE AND SILICA SOL-GEL ENCAPSULATED LACCASE EXTRACT, FROM *CORIOLUS HIRSUTUS*, IN SOLVENT-FREE MEDIA

6.1. Abstract

Using selected substrate models, the biocatalysis of free and sol-gel encapsulated laccase extract, from *Coriolus hirsutus*, in solvent-free media (SFM) was optimized in terms of enzymatic protein content, reaction temperature and agitation speed. Using FA as a substrate model, the encapsulated laccase in SFM effectively catalyzed its oxidation and resulted in a significantly ($P \leq 0.05$) increased enzyme specific activity of 238.19 nmol ferulic acid/µg protein/min as compared to that obtained in the aqueous one (83.88 nmol ferulic acid/µg protein/min). However, the free laccase extract did not show any enzymatic activity in SFM. The encapsulated laccase exhibited its highest enzyme specific activity in SFM (1,309.51 nmol ferulic acid/µg protein/min), with an optimum protein content of 2 µg, 150 rpm agitation speed and 60°C reaction temperature. The experimental findings also demonstrated that the use of a lower enzymatic protein content consistently resulted in a relatively higher laccase specific activity at all investigated temperatures of 25, 50 and 70°C. The kinetic studies indicated a K_m value of 0.17, 2.35, 7.67 and 4.62 µmol for the substrate models 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), FA, chlorogenic acid and gallic acid, respectively, with a corresponding k_{cat} value of 2.84, 4.72, 4.05 and 2.37 min^{-1}.

6.2. Introduction

The biocatalytic approach for the production of novel natural products relies on the versatility and the metabolic diversity of enzymes (Betancor and Luckarift, 2008). In comparison to the conventional chemical methods, enzyme-catalyzed reactions are “greener” alternatives and offer several advantages, including mild reaction conditions of temperature, pH and pressure as well as higher substrate stereo and regio-selectivity, increased product purity and energy efficiency (Chaibakhsh *et al*., 2009; Ghaffari-Moghaddam *et al*., 2015). Despite the advantages of enzyme-catalyzed processes, enzymes are sensitive to environmental reaction conditions of temperature and pH; nevertheless, the immobilization of enzymes can overcome these limitations and hence promote their use in various industrial processes (Betancor and Luckarift, 2008). In general, the

selection of an optimum solvent medium is essential for the stability of both enzyme and product, where it must be selected in terms of its compatibility with the biocatalyst, density, surface tension, toxicity and cost (Ghaffari-Moghaddam *et al*., 2015). In large-scale industrial processes, solvents are more often used as the media to transport the solid materials and to dissolve the immiscible reagents. In addition, solvents could also dissipate heat in the reaction medium and offer better selectivity (Yadav and Thorat, 2012). Nevertheless, solvents are major sources of volatile organic compounds and hence there is growing interest to develop novel strategies for the reduction of their use and to promote the use of more environmentally-friendly reaction media (Mack and Muthukrishnan, 2012; Yadav and Thorat, 2012).

In order to avoid the limitations, associated with the use of organic solvent systems in biocatalysis, solvent-free media (SFM) have been considered as the optimum choice in green chemistry. SFM not only lower the environmental impact of chemical processes, but also reduce their production costs and reaction times (Torregrosa *et al*., 2016). Due to the absence of organic solvents, these reaction systems facilitate the downstream processing and eliminate the need for solvent disposal and handling, thereby increasing the overall economic viability of the process (Yadav and Thorat, 2012).

The present study is part of an ongoing research program in our laboratory (Gill *et al*., 2018a,b) aimed at the biocatalysis in SFM of sol-gel encapsulated laccase enzymatic extract, from *Coriolus hirsutus*. The effects of selected parameters, including enzymatic protein content, reaction temperature and agitation speed have been investigated. In addition, the kinetic parameters of the laccase enzymatic extract biocatalysis were also determined with the use of selected phenolic substrate models.

6.3. Materials and Methods

6.3.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), FA, chlorogenic acid, gallic acid, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Mannitol was obtained from BDH Inc. (Toronto, ON). Tetraethyl orthosilicate (TEOS) (98% purity) and all other

chemicals of HPLC grade or higher were obtained from Fischer Scientific (Fair Lawn, N. J.). The anhydrous ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). All reagents and buffers were prepared using Milli Q plus (Millipore) water.

6.3.2. Production and recovery of laccase extract from Coriolus hirsutus

Laccase, from *Coriolus hirsutus* biomass, was extracted as per the protocol described by Taqi (2012). The enriched enzymatic extract was then lyophilized in the presence of mannitol (2.5% w/v) and stored at -80°C for further analysis.

6.3.3. Silica sol-gel encapsulation of the laccase enzymatic extract

The encapsulation of laccase extract in silica sol-gel was carried out as per Gill *et al*. (2018a,b), with modifications. Briefly, tetraethylorthosilicate (TEOS), deionized water and hydrochloric acid (HCl) were homogenized, using a water to silane molar ratio (*r*) of 6.78 and an HCl content of 3.22 µmol, with the PowerGen 125 homogenizer (KIA; Wilmington, N.C.). The homogenized components were subsequently stirred until a clear and elastic silica sol was obtained. For gelation, an aliquot of laccase extract suspension (0.86 mg protein/mL sol) prepared in potassium phosphate buffer (0.01 M, pH 6.0), was introduced to the silica sol, which was followed by an immediate addition of the potassium phosphate buffer (0.4 M, pH 7.0) with continuous agitation, until the onset of gelation (1 to 2 min). The resulting gel was aged for 24 h at 4ºC and later crushed, washed thoroughly and recovered by vacuum filtration using Whatman filter paper (No. 1). The gel was then lyophilized for 6 h at -42°C and stored under refrigeration to maintain its catalytic activity. All washing solutions were collected for protein determination.

6.3.4. Protein determination

The protein content of the free enzyme extract and washing solutions was determined according to a modification to the Lowry method (Hartree, 1972), using bovine serum albumin (BSA) for the calibration curve.

6.3.5. Enzyme activity

The enzyme activity assay for both free and encapsulated laccase enzymatic extract was carried out according to Bou-Mitri (2013). The enzyme activity was measured in sodium acetate buffer (0.1 M, pH 5.0) by following the laccase catalyzed oxidation of FA (0.03 M) at 50°C; the decrease

in the absorbance was measured spectrophotometrically, at 320 nm (Beckman Instruments Inc., San Raman, CA). All reactions were performed in triplicate trials and were run simultaneously with a blank reaction containing everything except the enzyme. The specific activities of the free and encapsulated laccase extract were defined as nmol converted substrate (ferulic acid/µg protein/min).

6.3.6. Biocatalysis of the encapsulated laccase in solvent-free media (SFM)

The biocatalysis of the free and encapsulated laccase extract was carried out in aqueous and solvent-free media (SFM). The reaction in the aqueous media was conducted in sodium acetate buffer (0.1 M, pH 5.0) as per the standard protocol for determining the laccase activity as described previously (Section 6.3.5). The laccase-catalyzed oxidation in SFM was carried out by the direct addition of the free or the encapsulated laccase extract (5 µg protein) and assayed for its enzyme activity, using FA (0.5 µmol) as a substrate. The substrate was prepared by its solubilisation in 40% (v/v) ethanol and subsequently dried by lyophilisation, before the addition of the enzymatic extract. The reaction mixture was then incubated at 50°C, with continuous agitation at 150 rpm, in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ). All reactions were run in triplicate trials with control reactions containing all components except the enzyme. The reaction was monitored over a period of 1 h and stopped at selected intervals of time by addition of 1 mL of cold-ethanol and subsequently centrifuged before measurement. The recovered samples were measured spectrophotometrically (Beckman Instruments Inc., San Raman, CA) at 320 nm to determine the initial oxidation rate. The specific activity was defined as nmol of converted ferulic acid/µg protein/min.

6.3.7. Effect of enzymatic protein content on laccase activity

The effect of enzymatic protein content, on laccase activity in SFM was investigated by varying the encapsulated protein from 0 to 7.0 µg; the enzymatic reaction was carried out using the standard protocol, described previously, at 50°C and 150 rpm.

6.3.8. Effect of reaction temperature on laccase activity

The effect of reaction temperature on laccase activity in SFM was determined, using a wide range of temperatures between 25 to 70°C. All enzymatic reactions were carried out according to the standard protocol described earlier, except that an optimized protein content of 2.0 µg was used.

6.3.9. Effect of agitation speed on laccase activity

The effect of varying agitation speeds on the enzymatic activity of laccase extract (50, 100, 150, 200 and 250 rpm) was investigated. All enzymatic reactions were conducted as per the standard protocol, using an optimized protein content of 2.0 µg and temperature of 60°C.

6.3.10. Kinetic parameters of sol-gel encapsulated laccase in SFM

The substrate specificity of the encapsulated laccase extract in SFM for the substrate models ABTS (ε_{420}, 36,000 cm^{-1}. M^{-1}), FA (ε_{320}, 14,547 cm^{-1}. M^{-1}), chlorogenic acid (ε_{320}, 26,322 cm^{-1}. M^{-1}) and gallic acid (ε_{270}, 9100 cm^{-1}. M^{-1}) was investigated. The substrates were prepared in the appropriate concentrations by their initial solubilisation in deionized water, except for FA that was prepared in 40 % (v/v) ethanol. All substrates were lyophilized prior to the reaction and addition of the encapsulated laccase. The kinetic parameters for the encapsulated enzyme were determined by measuring the initial velocity of the enzymatic reactions. The K_m and V_{max}, were calculated from the Lineweaver-Burk plots, while the turnover number, k_{cat} was determined as V_{max}/K_m. All reactions were conducted in triplicates.

6.3.11. Statistical analysis

All data were expressed by means of triplicate trials and their respective standard deviations (SD). The relative standard deviation (RSD) was calculated as the SD divided by the mean multiplied by 100. Correlation analyses were conducted using Microsoft-Excel (Microsoft) and the data were analyzed using PROC ANOVA, with a post-hoc comparison using Tukey's honest significance test. Values of $P \leq 0.05$ were considered significant.

6.4. Results and Discussion

6.4.1. Biocatalysis of laccase in solvent-free media (SFM)

The biocatalysis of free and encapsulated laccase extracts was investigated in aqueous and SFM. The results presented in Table 6.1 show that the relative laccase activity for the encapsulated enzyme extract in SFM was 283.97% as compared to that in the aqueous one. The absence of laccase activity for the free enzymatic extract in SFM could be due to the lack of an essential amount of required water for the maintenance of the enzyme conformation and function (Ghamgui *et al*., 2004).

Table 6.1. Effect of reaction medium type on the specific activity of encapsulated laccase extract, from *Coriolus hirsutus*, assayed with ferulic acid, at 50°C.

	Apparent laccase specific activity[a]		Relative laccase activity (%)[b]	
Reaction medium	Free	Sol-gel laccase	Free	Sol-gel laccase
Aqueous[c]	18.39 (9.66)[f]	83.88 (6.95)[f]	100 (9.66)[f]	100 (6.95)[f]
Solvent-free[d]	n.d.[e]	238.19 (6.14)	-	283.97 (5.02)

[a]Apparent laccase specific activity is expressed in nmol/µg protein/min.

[b]Relative laccase activity was defined as the specific activity of free or sol-gel encapsulated laccase extract, in a given reaction medium divided by that of the aqueous medium, multiplied by 100.

[c]The aqueous medium was composed of 100% sodium acetate buffer (0.1 M, pH 5.0).

[d]The solvent-free medium consisted of the sol-gel encapsulated laccase extract (5 µg protein) and lyophilized ferulic acid (0.5 µmoles).

[e]n.d. stands for not detected.

[f]Relative percentage standard deviation was defined as the standard deviations of triplicate trials divided by their respective means, multiplied by 100.

The water activity (a_w) for the free and encapsulated laccase was 0.46 and 0.92, respectively; hence, the significantly ($P \leq 0.05$) higher a_w for the encapsulated enzymatic extract may have resulted in an increased catalytic activity. In contrast, the free enzyme extract did not show any catalytic activity in SFM. The biocatalysis of the encapsulated laccase in SFM led to a significant ($P \leq 0.05$) enhancement in its specific activity (238.19 nmol/µg protein/min) as compared to that in the aqueous one (83.88 nmol/µg protein/min).

Previous study carried out in our laboratory, had succeeded in the biocatalysis of laccase enzymatic extract in aqueous and organic solvent media (OSM), where it was reported that laccase effectively catalyzed the oxidation of syringaldazine in the sodium acetate buffer and ternary micellar system (TMS) (Bou-Mitri, 2013). In addition, the results indicated that the free laccase enzymatic extract demonstrated a lack of catalytic activity in the monophasic hexane and biphasic acetate buffer/hexane (1:99, v/v) systems (Bou-Mitri, 2013); the relative laccase activity in TMS was found to be 84% as compared to that in the aqueous one. Bou-Mitri (2013) also reported that the laccase enzymatic extract, encapsulated in calcium alginate beads, resulted in an enhanced enzyme specific activity in monophasic and biphasic systems as well as the TMS with 1.1, 1.0 and 0.8 mmol converted ferulic acid/mg encapsulated protein/min, respectively, as compared to 0.6 mmol converted ferulic acid/mg encapsulated protein/min for the aqueous one.

Ghamgui *et al.* (2004) reported the biocatalytic synthesis of 1-butyl oleate by an immobilized lipase, from *Rhizopus oryzae*, in *n*-hexane and in SFM; these authors indicated that the initial water content present in the substrate or the enzymatic preparation was sufficient for maintaining the enzyme activity and any additional water could have resulted in a decrease in the substrate bioconversion in both media.

Ghaffari-Moghaddam *et al.* (2015) indicated that the biocatalysis of enzymes in SFM resulted in higher reaction rates and enhanced product yields; however, such enzymatic reactions were also associated with the denaturation of enzymes at high substrate concentrations, particularly polar organic compounds. In addition, the authors (Ghaffari-Moghaddam *et al*., 2015) suggested that these limitations may be overcome by the immobilization of enzymes. Similarly, Walsh *et al.* (2007) indicated increased levels of enantio-selectivity and activity for the enzymatic catalytic reactions, carried out in SFM. Bezbradica *et al.* (2006) reported that the maximum initial rate of

the lipase-catalyzed synthesis of amyl isobutyrate was relatively higher in SFM as compared to that in the organic solvent.

6.4.2. Effect of enzymatic protein content on laccase activity

Figure 6.1 illustrates the effect of the enzymatic protein content in the encapsulated laccase on its specific activity in SFM. The results (Fig. 6.1) show that the laccase enzyme activity decreased with a concomitant increase in the enzymatic protein content, where the highest specific activity (807.01 nmol/µg protein/minute) was obtained with 2 µg encapsulated protein. In order to confirm that the relatively higher laccase specific activity was obtained at a lower protein content, the effect of selected enzymatic protein content (2.0, 3.0, 4.0, 4.5 and 7.0 µg) was investigated at temperatures of 25, 50 and 70°C (Fig. 6.2). The overall experimental findings confirmed that the use of a lower protein content resulted consistently in a relatively higher laccase specific activity at all investigated temperatures.

The increased enzymatic specific activity, obtained at lower values of enzymatic protein, could be due to the saturation of the enzyme active sites by the substrate molecules (Yadav and Thorat, 2012). Under such conditions, any further increase in the protein content would have no effect on the laccase activity and this could be due to the absence of available substrate molecules, resulting hence in unoccupied active sites and a simultaneous decrease in the enzyme's catalytic activity (Yadav and Thorat, 2012). Based on the experimental findings (Fig. 6.2), a protein content of 2 µg was considered as the optimum amount for use throughout the study of all investigated parameters.

Similarly, Sun *et al.* (2012) reported that the substrate bioconversion yield in the lipase-catalyzed transesterification of coconut oil in SFM increased concomitantly with higher enzyme protein loadings; however, the presence of excessive enzyme particles resulted in mass-transfer limitations. Sun *et al*. (2012) also suggested that at higher enzymatic loadings, there was an increase in the viscosity of the reaction medium which resulted in a less effective substrate transfer because of the reduced interfacial area. Gómez *et al.* (2011) indicated that the reaction yield for the lipase-catalyzed synthesis of polyglycerol polyricinoleate in SFM was significantly improved with an initial increase in enzyme loading, up to a certain value, where beyond it, there was no additional enhancement.

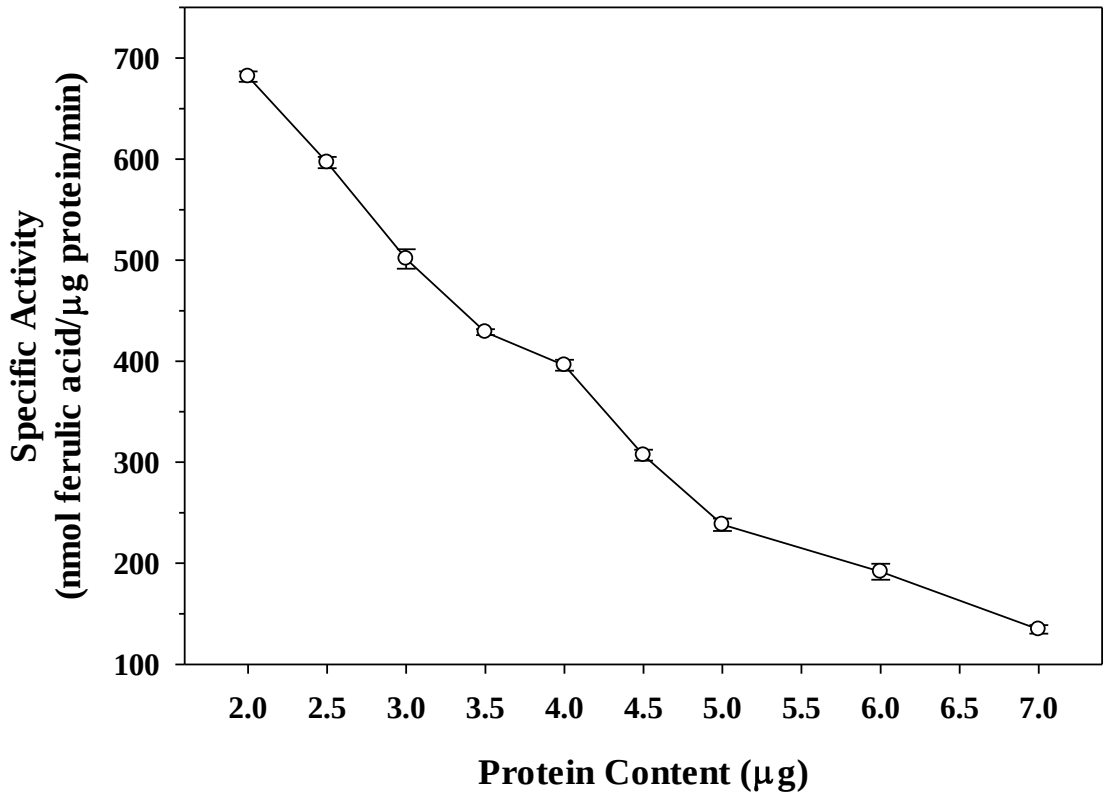


Figure 6.1. Effect of enzymatic protein content on the specific activity (○) of encapsulated laccase in solvent-free media (SFM), using 0.5 µmol of ferulic acid as a substrate. All results were determined by means of triplicate trials with the relative standard deviations between 3 and 8%.

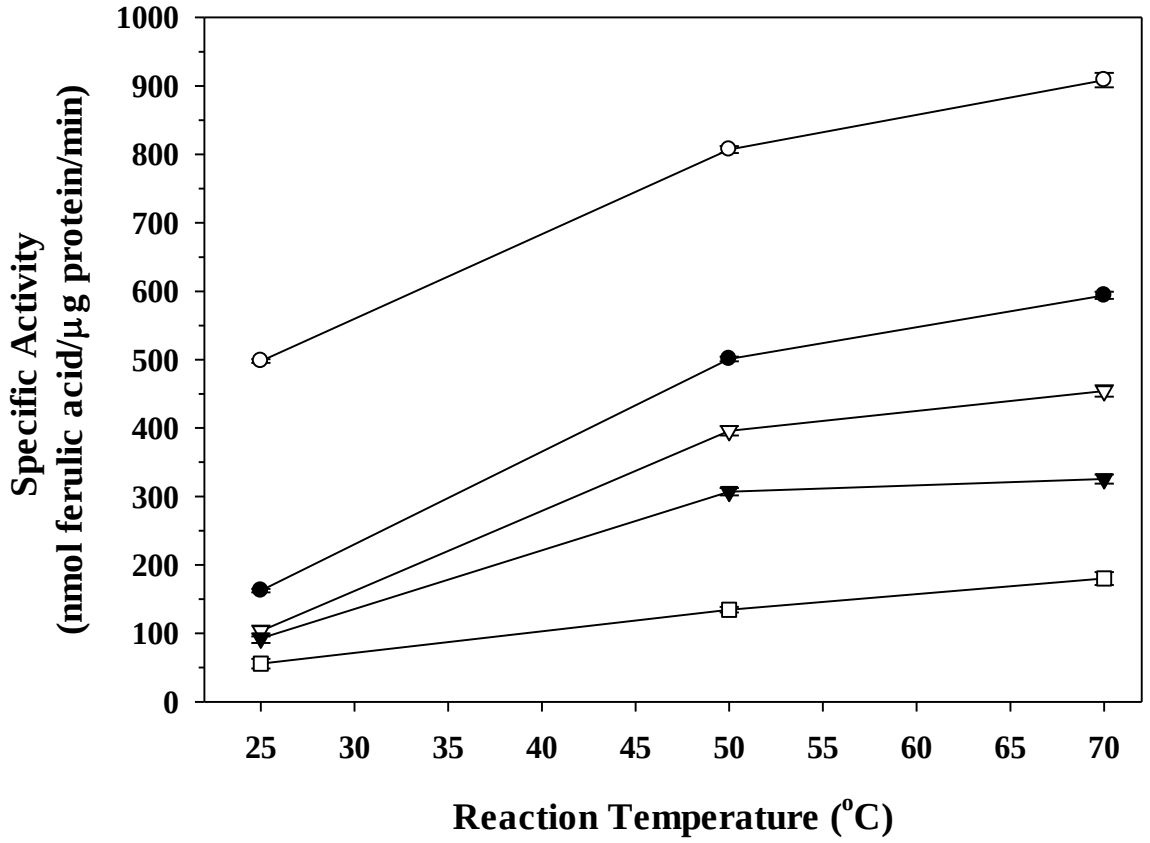


Figure 6.2. Effect of reaction temperatures on 2.0 (○), 3.0 (●), 4.0 (Δ), 4.5 (▲) and 7.0 µg (□) enzymatic protein content on the specific activity of encapsulated laccase in solvent-free media (SFM), using 0.5 µmol of ferulic acid as a substrate. All reactions were conducted in triplicates and the relative standard deviations vary between 2 and 11%.

6.4.3. Effect of reaction temperature on laccase activity

The effect of reaction temperature was investigated on the specific activity of the encapsulated laccase in SFM. The results presented in Figure 6.3 show that the laccase specific activity increased simultaneously with the increase in the reaction temperature up to 60°C; however, any further increase in temperature resulted in a decrease in the enzyme activity which could be attributed to the deactivation of the enzyme (Yadav and Thorat, 2012). The experimental findings indicate that a temperature of 60°C is optimum for the encapsulated laccase in SFM and it was used for the further study of other investigated parameters (Fig. 6.3).

Serrano-Arnaldos *et al.* (2016) investigated the effect of temperature on the solvent-free biocatalytic production of cetyl esters and indicated that higher reaction temperatures resulted in enhanced reaction rates, substrate diffusion and reduced substrate viscosity, hence improved mass-transfer rates. Sun *et al.* (2012) reported that although an increase in the reaction temperature resulted in an improved reaction rate, any further increase resulted in the reduction of the enzyme stability and led to its deactivation. Furthermore, Gómez *et al.* (2011) investigated the effect of the reaction temperature on the biocatalysis in SFM and suggested that this influenced the rate of the enzymatic reaction, evaporation of water from the reaction mixture and reaction viscosity; in addition, these authors reported that the use of a temperature higher than 50°C resulted in an adverse effect on the enzymatic activity which may be due to the loss of the native structural conformation of the enzyme.

6.4.4. Effect of agitation speed on laccase activity

Figure 6.4 illustrates the effect of agitation speed (50 to 250 rpm) on the laccase specific activity in SFM of the encapsulated enzymatic extract. The enzymatic reactions carried out at an agitation speed of 50, 100, 150, 200 and 250 rpm, resulted respectively in a laccase specific activity of 733.08, 1146.61, 1309.51, 1171.67 and 1015.03 nmol/µg protein/min. The experimental findings suggest that an increase in the agitation speed, up to 150 rpm, resulted in a concomitant increase in the encapsulated laccase specific activity; however, beyond this speed there was no increement in the enzymatic activity, thus indicating that the mass-transfer limitation may have been reduced to a minimum level (Sun *et al*., 2012).

Gawas *et al.* (2016) investigated the effect of agitation speed on the lipase-catalyzed synthesis in SFM of ethyl laurate and indicated that the effective mixing of reaction components in such conditions could overcome the mass-transfer limitations and may have resulted in the enhanced reaction rates; however, beyond a certain agitation speed a slight decrease was obtained. Gawas *et al.* (2016) suggested that the decrease in the reaction rate at relatively higher agitation speeds was due to the mechanical damage suffered by the enzyme molecules that may be caused by high shearing forces. Similar findings were reported by Sun *et al*. (2012) and Yadav and Thorat (2012), where the authors suggested that proper agitation at an optimum speed, up to a certain point could result in an increase in the enzymatic activity due to an enhanced mass-transfer to the surface of the enzyme particles. However, beyond this agitation speed an absence in the mass-transfer resistance may lead to a constant reaction rate (Yadav and Thorat, 2012).

6.4.5. Kinetic parameters of sol-gel encapsulated laccase in SFM

The enzymatic oxidation in SFM of selected phenolic compounds by the encapsulated laccase enzymatic extract was investigated (Table 6.2), using the optimum reaction conditions of 2 µg protein content, an agitation speed of 150 rpm and a 60°C of reaction temperature. For all investigated substrates, the Lineweaver-Burk plots of $1/v$ versus 1/[S] displayed a linear relationship, indicating a Michaelis-Menten kinetic behavior. The results show that the K_m value was 0.17, 2.35, 7.67 and 4.62 µmol for ABTS, FA, chlorogenic acid and gallic acid, respectively, with corresponding V_{max} value of 0.47, 11.11, 31.07 and 10.95 µmol/µg protein/min. The encapsulated laccase extract exhibited a relatively higher k_{cat} (4.72 min^{-1}) for FA as compared to that for chlorogenic acid (4.05 min^{-1}), ABTS (2.84 min^{-1}) and gallic acid (2.37 min^{-1}). The lower K_m values for ABTS and FA may indicate their favored binding to the laccase as compared to that with chlorogenic and gallic acids. As for the V_{max} values, a lower reaction rate may be attributed to the limited diffusion of the substrate to the enzyme active site (Jia *et al*., 2013). The overall experimental findings (Table 6.1) indicate that the laccase catalytic activity could be enhanced in SFM as compared to that in the aqueous one.

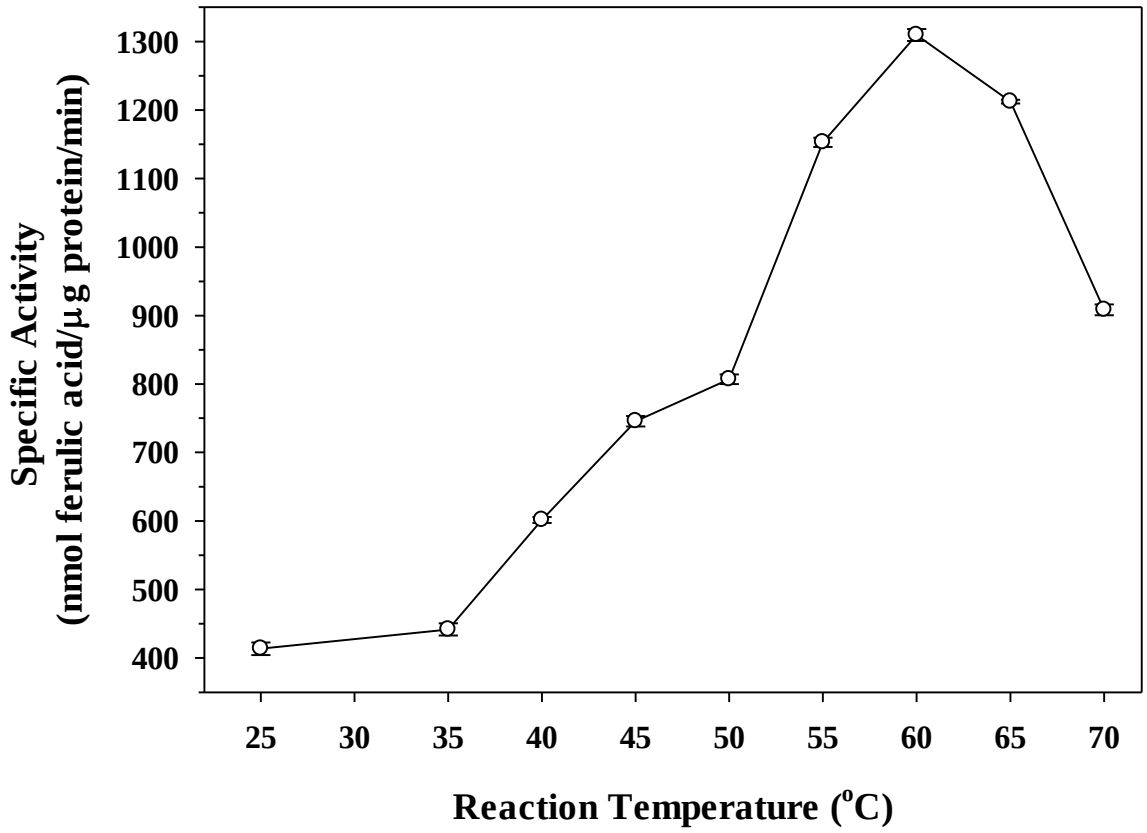


Figure 6.3. Effect of reaction temperature on 2.0 µg enzymatic protein content on the specific activity (○) of encapsulated laccase, in solvent-free media (SFM), using 0.5 µmol of ferulic acid as a substrate. All results were means of triplicate trials and the relative standard deviations vary between 2 and 9%.

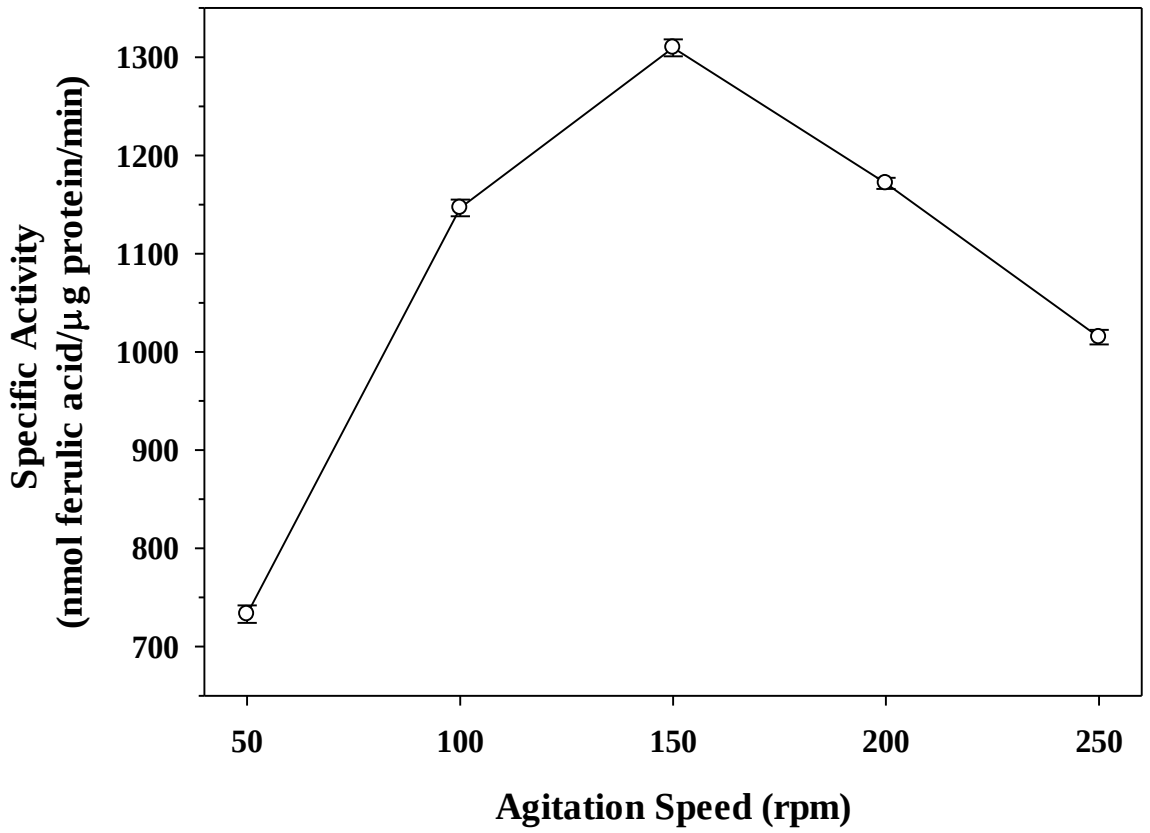


Figure 6.4. Effect of agitation speed on the specific activity (○) of encapsulated laccase extract, in solvent-free media (SFM), using 2 µg enzymatic protein, 0.5 µmol of ferulic acid and a reaction temperature of 60°C. All reactions were conducted in triplicates, with the relative standard deviations between 5 and 9%.

Table 6.2. Substrate specificity of sol-gel encapsulated laccase in solvent-free medium (SFM), using the optimum conditions of an enzymatic protein content of 2 µg, agitation speed of 150 rpm and 60°C reaction temperature.

Substrate	K_m [a]	V_{max} [b]	k_{cat} [c]	k_{cat}/K_m [d]
ABTS [e]	0.17 (7.88) [f]	0.47 (6.05)	2.84 (2.38)	17.21 (10.46)
Ferulic acid	2.35 (1.50)	11.11 (1.33)	4.72 (0.17)	2.07 (5.44)
Chlorogenic acid	7.67 (8.03)	31.07 (8.32)	4.05 (0.29)	0.51 (7.44)
Gallic acid	4.62 (5.43)	10.95 (2.79)	2.37 (3.43)	0.51 (8.36)

[a]The Michaelis-Menten constant K_m, was expressed in µmol.

[b]V_{max}, the apparent maximum velocity of laccase was defined as the µmol converted substrate/µg encapsulated protein/min.

[c]Turnover number, k_{cat} was defined as the ratio of K_m/V_{max}.

[d]The catalytic efficiency was expressed as the ratio of k_{cat}/K_m.

[e]ABTS stands for 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt.

[f]Relative standard deviation (%) was determined by means of triplicate trials.

In accordance with the current results, Bou-Mitri (2013) reported that the laccase enzymatic extract, obtained from *C. hirsutus*, encapsulated in calcium alginate beads demonstrated an enhanced specificity activity in organic monophasic, biphasic and TMS as compared to the aqueous one, with a K_m of 3.7, 4.6, 3.2 and 2.2 mM, respectively and a corresponding V_{max} of 21.4, 15.6, 7.8 and 4.6 mmol converted ferulic acid/mg encapsulated protein/min, with a k_{cat} of 5.8, 3.4, 2.4 and 2.1, respectively.

6.5. Conclusion

Using selected substrate models, an efficient method was developed for the biocatalysis in SFM of the sol-gel encapsulated laccase. The encapsulated laccase extract demonstrated an enhanced catalytic activity in SFM as compared to that in the aqueous one; however, the free enzyme extract did not show any catalytic activity in SFM. The laccase specific activity decreased with a concomitant increase in the enzymatic protein content. In addition, an increase in the reaction temperature and agitation speed, up to a certain point, resulted in a concomitant increase in the laccase specific activity. The kinetic studies demonstrated a Michaelis-Menten behavior for all selected substrate models, where the encapsulated laccase extract showed a relatively higher k_{cat} for FA as compared to other substrates.

CONNECTING STATEMENT TO CHAPTER VII

The biocatalysis of sol-gel encapsulated laccase enzymatic extract was effectively carried out in solvent-free media (SFM), where an enhanced catalytic activity was obtained as compared to that in the aqueous one (Chapter VI). Chapter VII presents the chemical structural characterization and the antioxidant capacity of the sol-gel encapsulated laccase-catalyzed end products, obtained in SFM.

CHAPTER VII

CHARACTERIZATION OF END PRODUCTS OF LACCASE-CATALYZED REACTION IN SOLVENT-FREE MEDIA, USING FERULIC ACID AS A SUBSTRATE MODEL

7.1. Abstract

Using ferulic acid (FA) as a substrate model, the characterization of the laccase-catalyzed end products obtained in solvent-free media (SFM) was investigated. The structural analyses of the end products were performed with size-exclusion chromatography (SEC), high-performance liquid chromatography (HPLC), spectrophotometric scanning, Fourier-transform infrared spectroscopy (FTIR) and electrospray ionization-mass spectrometry (ESI-MS). The HPLC chromatogram showed that the biocatalysis of laccase in SFM resulted in the synthesis of five end product fractions, with corresponding molecular weights (MW) of 1267.04, 880.06, 226.70, 159.74 and 132.25 Da; whereas their FTIR analysis confirmed the oligo/polymerization of FA via the C-O-C and C-C coupling modes. Further analysis of the end product fractions using ESI-MS in the negative ion mode, indicated the oligomerization of FA into di-, tri- and tetramers. In addition, the oligomerization of FA resulted in a 1.12 and 1.07-fold increase, respectively, in 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity and oxygen-radical absorbance capacity as compared to those of its monomer.

7.2. Introduction

Phenolic compounds are secondary metabolite derivatives that are produced during the pentose phosphate, shikimate and phenylpropanoid pathways in plants (Lin *et al*., 2016). These compounds are known for their free radical scavenging capacity, where they have been linked to numerous health benefits (Van Hung, 2016; Riebel *et al*., 2017). Hydroxycinnamic acid derivatives are a major group of plant polyphenols that are abundant in various cereals, fruits, vegetables as well as some beverages (Maurya and Devasagayam, 2010). FA (4-hydroxy-3-methoxy-phenyl)-acrylic acid is a ubiquitous cinnamic acid derivative that is known for its effective antioxidant capacity which is linked to the presence of the resonance stabilized phenoxy radical, involved in the termination of free radical chain reactions (Kumar and Pruthi, 2014). The antioxidant capacity of FA has promoted its applications in food, cosmetics and pharmaceutical industries (Aljawish *et al*., 2016).

Several studies have been reported on the beneficial effects of low molecular weight oligophenols, ranging from dimers upto octamers, as compared to those of their higher polymers (Lotito and Frei, 2006; Adelakun *et al*., 2012a,b; Bou-Mitri, 2013; Nemadziva *et al*., 2018). Moreover, oligophenols are characterized by a relatively higher absorption and lower or non metal and protein complexation property (Desentis-Mendoza *et al*., 2006). Various oligophenols have also demonstrated an enhanced superoxide scavenging activity as well as increased anti-inflammatory and anti-microbial activities as compared to those of their respective monomers (Elegir *et al*., 2008; Adelakun *et al*., 2012a,b; Bou-Mitri, 2013; Farhoosh *et al*., 2016). The antioxidant capacity of phenolic compounds is attributed to their structural characteristics; however, variations in the different electron-donating or withdrawing groups at various positions in the phenolic ring could lead to an improvement in their free radical scavenging capacity (Farhoosh *et al*., 2016).

Laccases (*p*-diphenol oxidase, E.C. 1.10.3.2) are known as 'green catalysts' due to their ability to oxidize a wide range of aromatic as well as some non-aromatic compounds using molecular oxygen as a cofactor, while producing water as the only by-product (Kudanga *et al*., 2017). The laccase-catalyzed oxidation reactions result in the generation of phenoxy radicals that may undergo further condensation reactions to form dimers, oligomers and higher polymers, covalently coupled by C-C and C-O linkages (Bou-Mitri, 2013; Nemadziva *et al*., 2018). In general, the structure and polymerization extent of the enzyme-catalyzed oxidation end products depends on the reaction media and their conditions as well as its substrate specificity (Kazandjian and Klibanov, 1985; Mustafa *et al*., 2005; Bou-Mitri, 2013).

This work is part of an ongoing research program in our laboratory (Gill *et al*., 2018a,b) aimed at the biocatalysis of encapsulated laccase enzymatic extract in solvent-free media (SFM) for the synthesis of oligomers from FA, with an enhanced antioxidant capacity. The overall objective of the current study was to investigate the structural and antioxidant characterizations of the synthesized end products.

7.3. Materials and Methods

7.3.1. Materials

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA) via Cedarlane Labs (Burlington, ON). FA, bovine serum albumin (BSA) 2,2'-

azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), α-tocopherol and tetrahydrofuran (THF)-HPLC grade were purchased from Sigma Chemical Co. (St. Louis, MO). Tetraethyl orthosilicate (TEOS) (98% purity) and all other chemicals of HPLC grade or higher were obtained from Fischer Scientific (Fair Lawn, N. J.). The anhydrous ethanol was purchased from Commercial Alcohols Inc. (Branchville, QC). All reagents and buffers were prepared in using Milli Q plus (Millipore) water.

7.3.2. Encapsulation of the laccase enzymatic extract from Coriolus hirsutus

The encapsulation of laccase extract in silica sol-gel was carried out following the optimized method as described in Chapter V (Section 5.3.3.). Briefly, tetraethylorthosilicate (TEOS), deionized water and hydrochloric acid (HCl) were homogenized with a water to silane molar ratio (*r*) of 6.78 and an HCl content of 3.22 µmol, using a PowerGen 125 homogenizer (KIA; Wilmington, N. C.). An aliquot of laccase extract suspension (0.86 mg protein/mL sol) prepared in potassium phosphate buffer (0.01 M, pH 6.0) was introduced to the homogenized sol, followed by an immediate addition of the potassium phosphate buffer (0.4 M, pH 7.0) with continuous agitation, until the onset of gelation (1 to 2 min). The gel was then aged for 24 h at 4ºC, crushed, washed and recovered by vacuum filtration using Whatman filter paper No. 1. The sol-gel encapsulated laccase enzymatic extract was later lyophilized for 6 h at - 42°C and stored under refrigeration to maintain its catalytic activity. All washing solutions were collected for protein determination.

7.3.3. Laccase-catalyzed oxidation of ferulic acid in solvent-free media (SFM)

The laccase-catalyzed oxidation of FA in SFM was performed as per the optimized protocol as described in Chapter VI (Section 6.3.6.). The biocatalysis in SFM was carried out by the direct addition of the encapsulated laccase extract (25 µg protein), using FA (2.5 µmol) as a substrate. The FA was prepared by its solubilization in 40% (v/v) ethanol and subsequent lyophilization prior to the addition of the encapsulated laccase enzymatic extract. The enzymatic reaction mixture was then incubated at 60°C, with continuous agitation at 150 rpm, in an orbital shaker (New Brunswick Scientific Co., Inc., Edison, NJ). The reaction was carried out for a period of 3 h and stopped at selected intervals of time by the addition of 1 mL of cold-ethanol. For the recovery of the end products, the samples were initially centrifuged, concentrated using nitrogen and re-solubilized in

40% (v/v) ethanol, subsequent to which they were lyophilized using a FreezeZone® stoppering tray dryer (Labconco; Kansas City, MS) for 48 h.

7.3.4. Protein determination

The protein content of the enzymatic fractions and washing solutions was determined according to a modification to the Lowry method (Hartree, 1972), using bovine serum albumin (BSA) for the calibration curve.

7.3.5. Characterization of the laccase-catalyzed end products

7.3.5.1. Characterization of the end products using SEC

The characterization of the laccase-catalyzed end products obtained in SFM was carried out as per the protocol described by Bou-Mitri (2013), using size-exclusion chromatography (SEC) with the conventional liquid chromatrography. The recovered end products (1 mg/mL) and FA (1 mg/mL) were solubilized in a solution of deionized water and methanol (1:1, v/v) and centrifuged (15,000xg, 10 min). The supernatant was applied to a Trisacryle GF05 M (Biosepra Inc.; Marlborough, MA) column (2.4x90 cm) and eluted using deionized water, at a flow rate of 0.4 mL/min. Fractions of 4 ml were collected and monitored at 280 and 320 nm, using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc; San Raman, CA). The recovered fractions were also characterized in terms of their respective K_{av} values, where $K_{av} = (V_e\text{-}V_0)/(V_t\text{-}V_0)$ such that K_{av} was defined as the partition co-efficient, V_e was the elution volume of the solute, V_0 was the column volume and V_t was the total permeable column volume.

7.3.5.2. Determination of molecular weight by HPLC

The molecular weight (MW) of the recovered end product fractions were analyzed with high-performance liquid chromatography (HPLC), using Beckman Gold system (Beckman Instrument Inc; San Raman, CA), equipped with a UV diode-array detector (DAD) (Beckman, model 168). Stock solutions (3 mg/mL) of polystyrene standards (Pressure chemical; Pittsburg, PA), with MW of 580, 1470, 1680 and 2400 Da, as well as a series of phenolic acids solutions (1 mg/mL) of catechol (110 Da), FA (194 Da) and catechin (290 Da), were prepared. The substrate and the end products (1.5 mg/mL) were solubilized in a mixture of tetrahydrofuran (THF)/methanol/H_2O (70:18:12, v/v/v). A 50 µL sample was analyzed on a polydivinilbenzene (Jordi Gel DVB) column (300x7.8 mm I.D.), purchased from Jordi Labs (Mansfield, MA), using an isocratic mobile phase

of a mixture of THF/methanol/H_2O (70:18:12, v/v/v). The flow rate of 0.15 mL/min and the detection at 254, 280 and 420 nm were carried out. All analyses were conducted in duplicate trials. In order to characterize the end product fractions, selected fractions were recovered from the HPLC analysis and concentrated, using the Automatic Environmental Speedvac System, and subsequently lyophilized with the use of a FreezeZone® stoppering tray dryer (Labconco; Kansas City, MS).

7.3.5.3. Spectrophotometric scanning of purified end product fractions

The substrate as well as purified end product fractions were solubilized in a mixture of THF/methanol/H_2O (70:18:12, v/v/v) and analyzed spectrophotometrically, within the range of 200 to 800 nm, using a Beckman DU-650 spectrophotometer.

7.3.5.4. FTIR analysis of purified end product fractions

The Fourier-transform infrared (FTIR) analysis of the purified end product fractions was performed as described previously by Bou-Mitri (2013) for the structural elucidation of the laccase-catalyzed end products in SFM. The recovered end product fractions and the substrate were placed on the ATR crystal and scanned at room temperature to obtain their infrared spectra, using a Bruker Alpha-P spectrometer (Bruker Optic GmbH; Ettlingen, GE) equipped with a detracted triglycine sulfate (DTGS) detector, a temperature-controlled single-bounce diamond ATR crystal and a pressure application device particularly for solid samples. Processing of the FTIR data was carried out using the Bruker OPUS software.

7.3.5.5. ESI-MS analysis of purified end product fractions

The purified fractions of the end products, obtained by the laccase-catalyzed oxidation of FA, were characterized by electrospray ionization-mass spectrometer (ESI-MS), using the Exactive Plus Orbitrap-based Fourier Transform-Mass Spectrometer (FT-MS) system (Thermo Fisher Scientific; San Jose, CA) and the LCQ-Advantage quadrupole-ion trap mass spectrometer system (Thermo Electron Corporation; San Jose, CA). The purified fractions were solubilized in methanol and each sample (5 μL) was introduced by direct loop injection in the ESI source. The mass spectrometer was operated in positive and/or negative ion mode, with a collision energy source of 3.8 KV at 300ºC capillary temperature and nitrogen auxiliary gas.

7.3.5.6. Antioxidant assays

7.3.5.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The antioxidant capacity of FA and the laccase-catalyzed end products was determined, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The reaction was performed by preparing 50 µL of the samples in 450 µL of Tris-HCl buffer (0.1 M, pH 7.4) to obtain a final volume of 500 µL, followed by the introduction of 1 mL of 0.1 mM DPPH solution into each sample. The control trial was carried out with 50 µL methanol, while 1 mL water was used as the blank. The reaction mixture was incubated for a period of 30 min at room temperature and the absorbance of each sample was determined spectrophotometrically with the Beckman DU-650 at 517 nm. A standard curve, using α-tocopherol (vitamin E analogue) of selected concentrations of 0.0, 2.5, 3.0, 5.0 and 10.0 mg/mL was also performed. The half maximal inhibitory concentration percentage ratio for the DPPH was calculated as follows:

$$\text{Half maximal inhibitory concentration (\%)} = \{(A_c - A_s)/A_c\} \times 100 \quad \text{(I)}$$

where, A_c and A_s are the absorbance of the control and sample, respectively. All reactions were carried out in triplicates.

7.3.5.6.2. Oxygen-radical absorbance capacity (ORAC) assay

The antioxidant capacity of FA and its laccase-catalyzed end products was also determined, with the oxygen-radical absorbance capacity (ORAC) method, which measured their free-radical scavenging capacity against peroxyl radicals. The assay was performed according to the protocol described previously by Garrett *et al*. (2010), with certain modifications. The reaction was carried out in potassium phosphate buffer solution (75 mM, pH 7.4), where solutions of the antioxidants (25 µL) and 150 µL of fluorescein (16 nM) were introduced into each well. The fluorescence readings were recorded for time 0 and the reaction was initiated by injecting 25 µL of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution (79.65 mmol/L) into the respective wells. The fluorescence was measured at 485 nm excitation and 520 nm emission wavelengths, until complete extinction. A blank reaction containing the phosphate buffer (25 µL) was performed in tandem with the assay. Calibration curve, using the 25 µL of the standard Trolox of 0.0, 6.25, 12.5, 25.0, 50.0 and 100.0 mM with a blank trial, was also carried out with fluorescein. The final results were calculated by comparing the net areas under fluorescein decay curves between the blank and end

products, using Microsoft excel. The ORAC value was expressed as μmol Trolox equivalent (TE)/g product. All reaction mixtures were prepared in triplicates.

7.4. Results and Discussion

7.4.1. Characterization of the SEC purified end products

The elution of the substrate FA and laccase-catalyzed end product obtained by size-exclusion chromatography (SEC), was monitored at 280 and 320 nm, where a single major peak was obtained (Fig. 7.1). In addition, using the Trisacryl GF05 gel, the elution of FA and the synthesized end products, resulted in an elution volume (V_e) of 160 and 192 ml, respectively, with a corresponding partition coefficient (K_{av}) of 0.35 and 0.43. The experimental findings indicate the presence of different MW for the end product as compared to that of the initial substrate.

Using the same Trisacryl GF05 gel column, Bou-Mitri (2013) reported a K_{av} value of 0.36, 0.47 and 0.50, respectively, for FA and its laccase-catalyzed end products in aqueous as well as in organic solvent media (OSM); the author suggested relatively identical MW for the synthesized end products in both reaction media. Similarly, Kermasha *et al*. (2002) obtained a K_{av} value of 1.38, 1.54, 1.65 and 1.85, respectively, for 4-methyl catechol (124.4 Da), *p*-cresol (108.1 Da), catechol (110.1 Da) and catechin (290.3 Da), where they indicated that the 4-methyl catechol and catechin were the first and last to elute, respectively, as compared to the other phenolic compounds; these authors suggested that such chromatographic behaviour could be due to the steric hinderance and hydrogen bond interactions of the separated compounds with the Trisacryl GF05 gel.

7.4.2. HPLC analysis of end product fractions

Figure 7.2 illustrates the high-performance liquid chromatography (HPLC) analysis of the laccase-catalyzed end products of FA as a substrate. The results show the presence of five end product fractions, EP-F1, EP-F2, EP-F3, EP-F4 and EP-F5, eluted at retention times (t_R) of 48.717, 53.800, 72.717, 77.600 and 80.233 min, respectively.

Using selected phenolic acids and polystyrene standards, the MW of the separated end product fractions was also determined by gel-permeation chromatography (GPC) HPLC. The results (Table 7.1) show that the MWs of the end product fractions, EP-F1, EP-F2, EP-F3, EP-F4 and EP-

F5, were 1,267.04, 880.06, 226.70, 159.74 and 132.25 Da, respectively, with corresponding degrees of polymerization (DP) of 6.8, 4.7, 1.2, 0.9 and 0.7, as compared to FA.

A previous study carried out in our laboratory (Bou-Mitri, 2013) indicated that the laccase-catalyzed oxidation of FA in aqueous medium resulted in three end product fractions, Aq-P1, Aq-P2 and Aq-P3 of 295.3, 445.7 and 739.8 Da, respectively, whereas that in hexane medium was of 321.4 Da, with corresponding DP of 1.5, 2.3, 3.9 and 1.7, as compared to that for FA.

Aljawish *et al*. (2014) investigated the reversed-phase (RP)-HPLC analysis of the enzymatic oxidation of FA, by a laccase from *Myceliophthora thermophila*, in aqueous medium, where only one major product, with a MW of 386 Da was characterized. Similarly, Adelakun *et al.* (2012a) reported that the RP-HPLC analysis of the laccase-catalyzed end products of FA in polar and biphasic OSM, resulted in three major products, attributed to two types of dimers (*m/z* 385.11) and a trimer (*m/z* 579.21); however, a tetramer was also obtained which was only detected by the MS analysis. In addition, Adelakun *et al*. (2012b) showed that the RP-HPLC analysis of the laccase-catalyzed oxidation of 2,6-dimethoxyphenol (DMP) in biphasic aqueous-organic media, resulted in the formation of five products, where the main product was a dimer of *m/z* 305.07.

7.4.3. Spectrophotometric scanning of the end product fractions

The spectrophotometric results presented in Figure 7.3 indicate that the absorbance of FA and its laccase-catalyzed end product fractions, EP-F1, EP-F2, EP-F3 and EP-F4, demonstrated three maximum absorbance (λ_{max}) at 240, 280 and 320 nm; however, a second λ_{max} at 220 nm was also obtained for EP-F1, EP-F2 and EP-F4. Moreover, EP-F5 showed one λ_{max} at 220 nm; the FA as well as all the end products absorbed in the region between 200 to 360 nm. The decrease and shift in the absorbance bands of the end product fractions as compared to that of FA could be due to the n-π* transition (n to pi star transition) resulted by the C-C and C-O-C coupling, attributed to phenolic side-chains reactions (Bozic *et al.*, 2012).

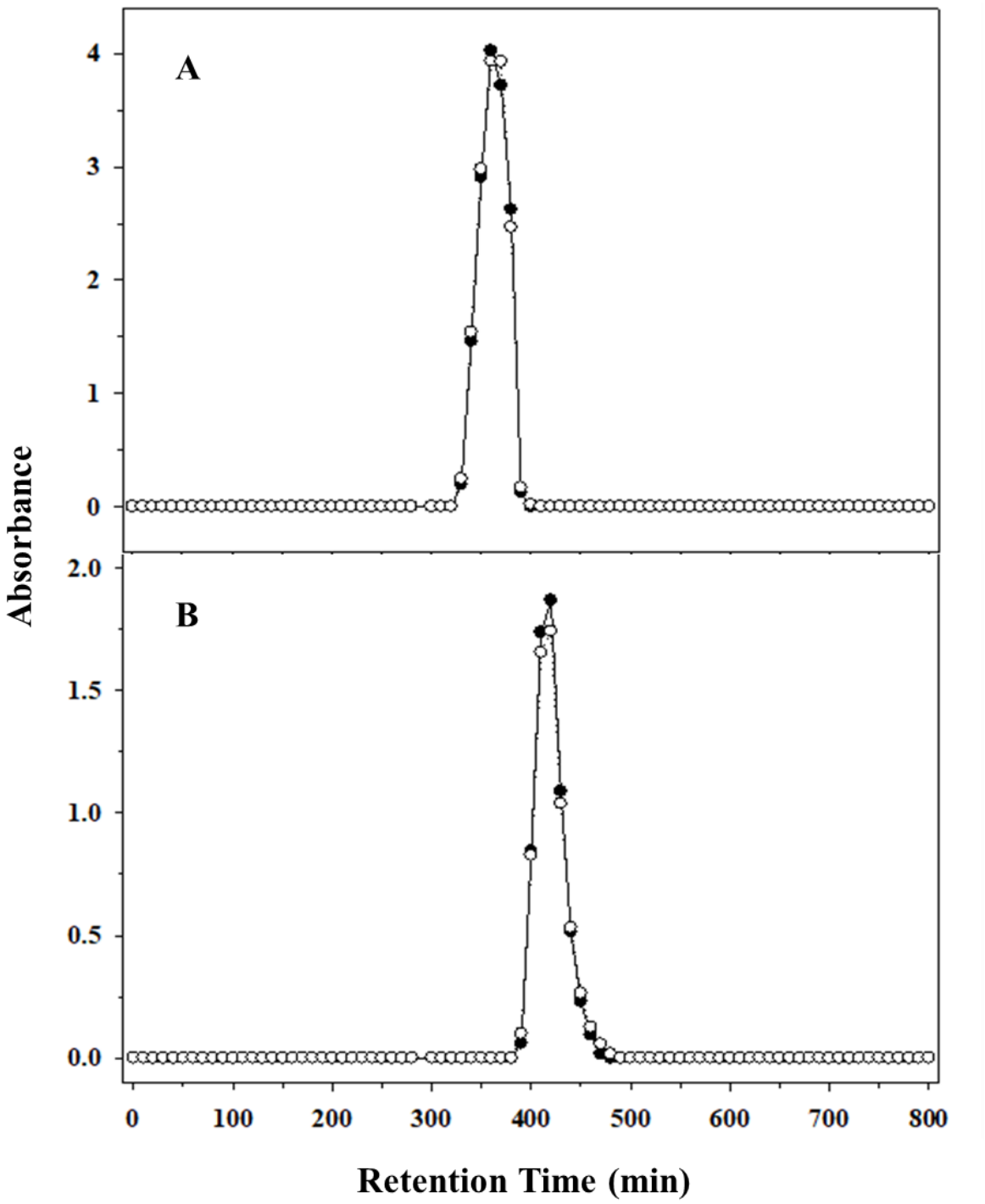


Figure 7.1. Size-exclusion chromatrography elution profiles of ferulic acid (A) and laccase-catalyzed end products (B) obtained in solvent-free media (SFM) with absorbance at 280 nm (○) and 320 nm (●).

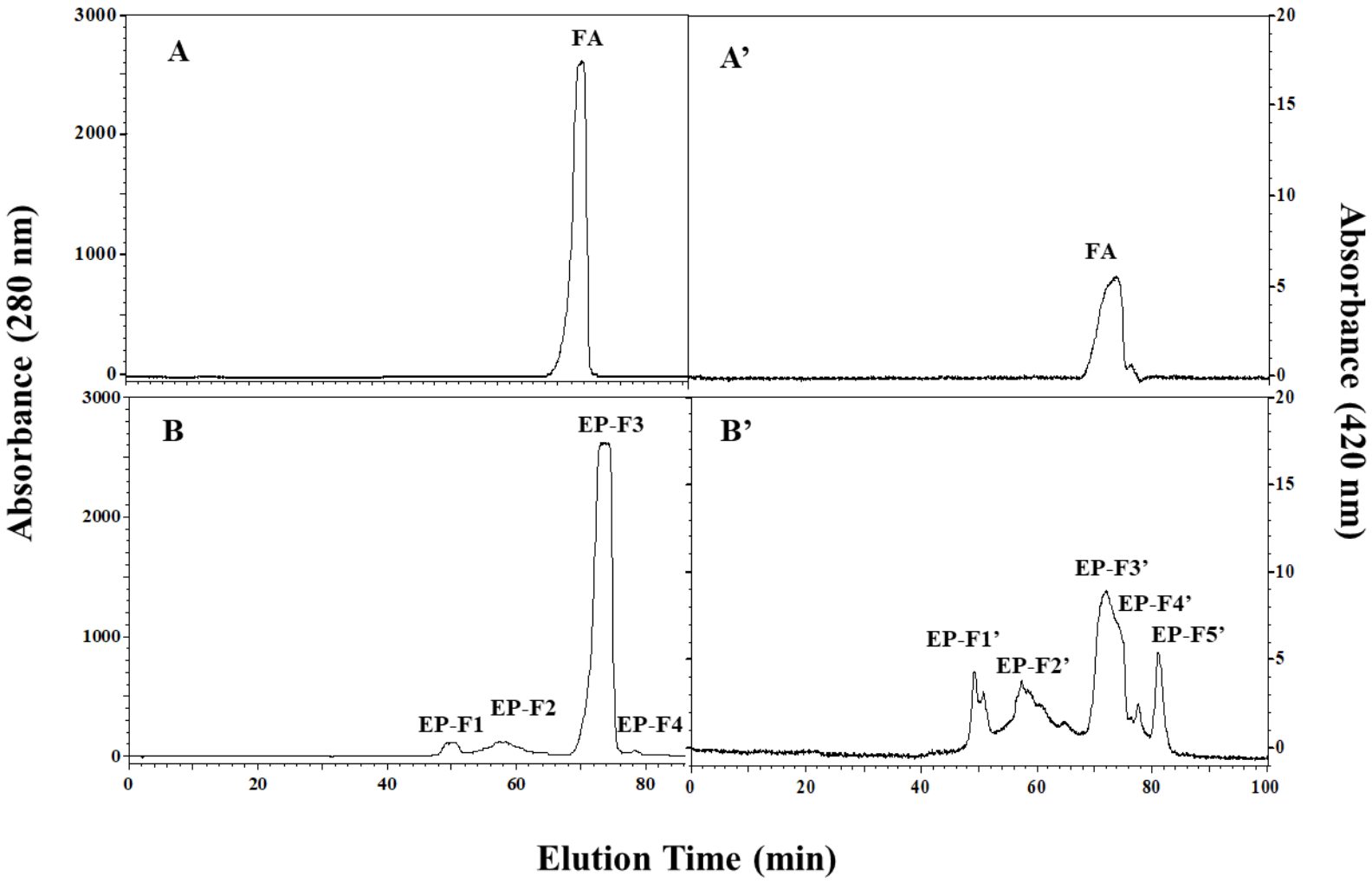


Figure 7.2. High-performance liquid chromatography (HPLC) elution profiles of ferulic acid (A and A') as well as its catalyzed end product fractions (B and B') obtained after 180 min in solvent-free medium (SFM), at an absorbance of 280 nm and 420 nm.

Mathew *et al*. (2007) reported the bioconversion of FA by an isolated yeast strain into an intermediate compound 4-vinyl guaiacol, where the decarboxylation of FA led to a hypochromic shift in a λ_{max} of 290 and 310 nm for FA to λ_{max} of 210 and 260 nm for that of the product; these authors concluded that this shift may be due to the formation of 4-vinyl guaiacol which was confirmed with the λ_{max} of vinyl guaiacol, generally reported at 265 and 226 nm. Furthermore, Aljawish *et al*. (2014) indicated that the laccase-catalyzed oxidation of FA and ethyl ferulate resulted, respectively, in an increase and a decrease in the absorbance at 322 and 230 nm; these authors suggested that the change in the spectra may be due to the formation of quinones, which could be caused by a decrease in the intensity of the long-wave bands, as a result of the dissociation of the carboxylic acid.

Bou-Mitri (2013) reported the spectrophotometric analysis of the laccase-catalyzed end products of FA, obtained in aqueous and OSM, where both of them demonstrated two λ_{max} at 280 and 320 nm; in addition, the end products obtained in aqueous medium also exhibited a λ_{max} at 420 nm. The author also indicated that FA absorbed in the region of 250 to 300 nm as compared to 250 to 350 nm for the end products (Bou-Mitri, 2013).

7.4.4. FTIR analysis of the end product fractions

In order to elucidate the structural differences between the FA and its laccase-catalyzed end products, Fourier-transform infrared (FTIR) analysis was performed. The results shown in Figure 7.4 indicate that the end product fractions exhibited different IR spectra, with the presence of relatively wide absorption bands, as compared to that of FA used as a substrate; however, the spectra for fractions EP-F4 and EP-F5 show identical profiles and may be considered as the same fraction or as isomers, eluted respectively, in very close elution times of 77.600 and 80.233 min. All fractions showed typical polyphenolic characteristics as a result of stretching of O-H, C=C and C-O/C-C groups within the regions, respectively, of 3200-3550, 1450-1600 and 1200-1300 cm^{-1} (Kupriyanovich *et al*., 2007; Bozic *et al*., 2012). In addition, the strong absorbtion at 1,033 cm^{-1}, attributed to the C-O stretching vibration bands, was split into two absorption bands for the fractions, EP-F1 (1,058, 1,034 cm^{-1}), EP-F3 (1,060, 1,033), EP-F4 (1,060, 1,035 cm^{-1}) and EP-F5 (1,059, 1,035 cm^{-1}) and three bands for EP-F2 (1,073, 1,054 and 1,021 cm^{-1}). The shift and split in these absorption bands may indicate an extended degree of polymerization, with C-C linkages between the phenolic acid (Bozic *et al*., 2012; Sun *et al*., 2013).

Table 7.1. High-performance liquid chromatography analysis of ferulic acid and its laccase-catalyzed end product fractions in solvent-free media (SFM).

Compound	t_R[a]	M.W[b]	D.P[c]
Ferulic acid	75.350	187.70	1.0
EP-F1[d]	48.717	1,267.04	6.8
EP-F2[d]	53.800	880.06	4.7
EP-F3[d]	72.717	226.70	1.2
EP-F4[d]	77.600	159.74	0.9
EP-F5[d]	80.233	132.25	1.7

[a]Retention time (t_R) was defined as the elution time for the product using the HPLC polydivinylbenzene (DVB) column (300x7.8 mm I.D.; Jordi Labs, Mansfield, MA), with isocratic elution of a mixture of THF/methanol/H_2O (70:18:12, v/v/v) with 0.15 mL/min flow rate.

[b]Molecular weight (M.W) was calculated from the retention time of each eluted standard compound in the GPC-HPLC.

[c]Degree of polymerization (D.P) was calculated from the molecular weight relative to that of the ferulic acid.

[d]EP-F1, EP-F2, EP-F3, EP-F4 and EP-F5 were the laccase-catalyzed end product fractions obtained by the HPLC analysis.

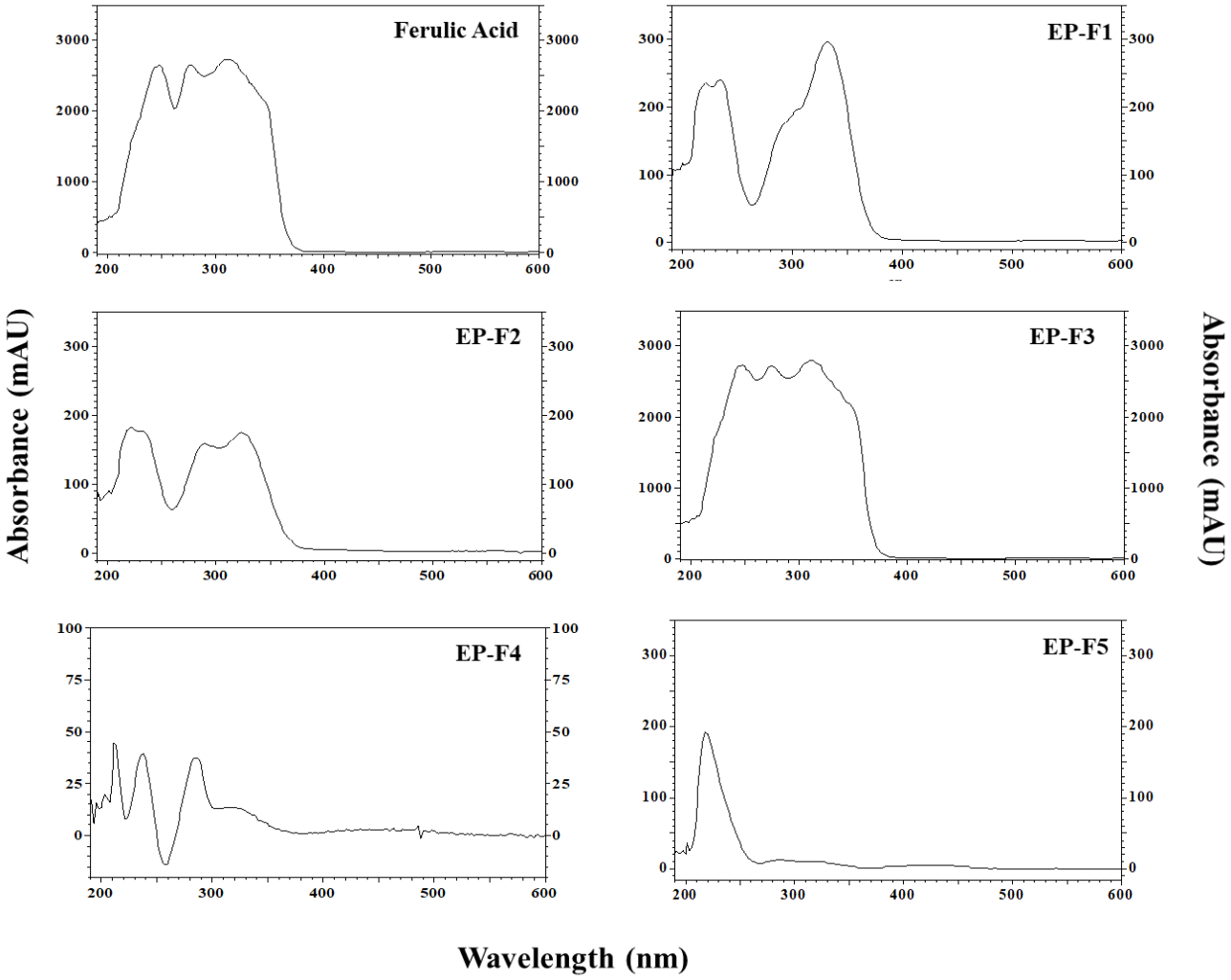


Figure 7.3. Spectrophotometric scanning profiles of ferulic acid as well as the laccase-catalyzed end product fractions obtained in solvent-free media (SFM) including, EP-F1, EP-F2, EP-F3, EP-F4 and EP-F5 solubilized in THF/methanol/H_2O (70:18:12, v/v/v).

In addition, the C=O vibration band at 1,687 cm^{-1}, corresponding to the carboxylic group of FA, was obtained at 1,722 cm^{-1} in the spectra for EP-F1 and EP-F2, whilst at 1,759 cm^{-1} in that of EP-F3; however, this band was absent for the fractions EP-F4 and EP-F5. The spectra for the end product fractions also resulted in strong absorption bands within 920 to 970 cm^{-1}, attributed to the *trans*=C-H out of plane bending as well as between 850 to 852 cm^{-1} for *p*-substituted aromatic rings (Karaki *et al*., 2017). Nazareth and Mavinkurve (1986) suggested that the IR analysis of *Fusarium solani* catalyzed degradation of FA showed the presence of hydroxyl, aromatic and vinyl double bond groups, within the regions of 3560-3520, 1615-1600 and 990-905 cm^{-1}, respectively

Bou-Mitri (2013) reported different FTIR spectra for the laccase-catalyzed end products of FA in aqueous and OSM, with O-H and C-O stretching vibration bands and were linked to an extended degree of polymerization; this author indicated the same linkage groups with different vibrational modes in both media, hence suggesting that the enzymatic oxidation proceeded differently in both media. Sun *et al*. (2013) reported the FTIR analysis of the laccase-catalyzed oxidation of selected phenolic compounds, including catechol, resorcinol and hydroquinone, where the characteristic O-H, C-H, C-O and C=O stretching vibrational bands were obtained for all polymers. Similarly, Bozic *et al*. (2012) indicated that the FTIR spectra of laccase-catalyzed oligomers/polymers of gallic acid showed two absorption bands at 1,025 and 1,069 cm^{-1}, while that for caffeic acid demonstrated a single one at 1,041 cm^{-1}, all linked to the C-C coupling.

7.4.5. ESI-MS analysis of the end product fractions

In order to characterize the molecular structure of the laccase-catalyzed end products obtained in SFM, further analysis of the fractions by the electrospray ionization-mass spectrometry (ESI-MS) in the negative ion modes was carried out (Fig. 7.5.). The analyses of the end product fractions (data not shown) showed the presence of a mixture of dimers (385.09 Da), trimers (579.23 Da) and tetramers (775.46 Da) and their fragment ions. The fragmentation pattern of the fraction EP-F1 (Fig. 7.5) shows peaks at mass to charge ratio (*m/z*) of 740.49 and 713.47, respectively, which could be attributed to a demethylated tetramer that resulted in a concomitant loss of H_2O $[4M\text{-}H^+\text{-}CH_3\text{-}H_2O]^{--}$ as well as a neutral loss of 2CH $[4M\text{-}H^+\text{-}2CH]^{--}$.

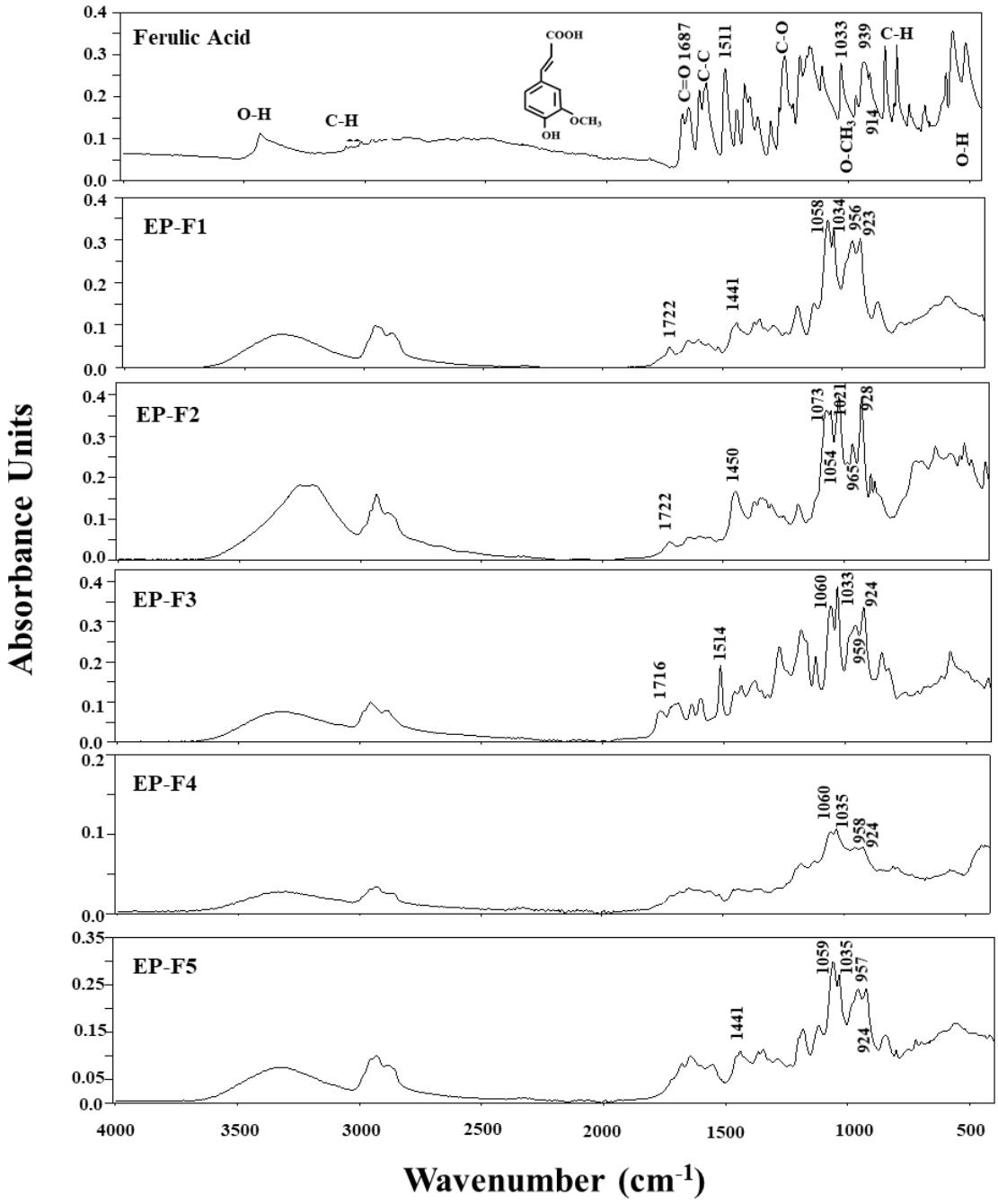


Figure 7.4. Fourier-transform infrared spectroscopy (FTIR) spectra of the ferulic acid and the laccase-catalyzed end product fractions, EP-F1, EP-F2, EP-F3, EP-F4 and EP-F5 in solvent-free media (SFM).

The results also indicate fragmentation patterns for the trimer at *m/z* of 565.32 [4*M*-H^+-CH]$^{--}$, 514.32 [4*M*-H^+-OCH_3-H_2O]$^{--}$, 487.30 [3*M*-H^+-2CH]$^{--}$, 393.27 [3*M*-H^+-CO_2-CH-2H_2O $^-$]$^{--}$, 371.96 [3*M*-H^+-H_2O]$^{--}$, 335.98 [3*M*-H^+-CH_3-H_2O]$^{--}$ and 255.23 [3*M*-H^+-2OCH_3-H_2O]$^{--}$. The spectrum for the end product fraction EP-F2, also shows a peak at 487.30, indicating the fragmented ion from the trimer; however, the results (not presented) also showed the presence of a dimer with its fragmented ions at 285.14 [2*M*-H^+-2CO_2-CH]$^{--}$, 229.12 [2*M*-H^+-CO-2CH]$^{--}$, 215.07 [2*M*-H^+-CH]$^{-}$ $^{-}$ and 181.06 [2*M*-H^+-CH_3-H_2O]. Similarly, the fragmentation pattern of the fraction EP-F3 indicates the decarboxylated and demethylated fragmented ions of the dimer as well as residual FA obtained at *m/z* of 193.04. The spectrum for the end product fractions EP-F4 and EP-F5 demonstrate identical mass spectrums, with abundant ions obtained at 180.97, 170.94 and 112.96 that could correspond to fragmentation ions produced from the dimer and/ or degradation of FA.

Previous study by Bou-Mitri, 2013 carried out in our laboratory reported similar ESI-MS results for alginate encapsulated laccase-catalyzed oxidation of FA, with fragmention ions of *m/z* 112.76, 248.61, 285.01, 371.08 that corresponded to the decarboxylated and demethylated mixture of FA oligomers; the author confirmed the formation of FA dimers (386.4 Da), trimers (578.5 Da) and tetramers (770.7 Da) in aqueous medium, while a dimer (386.4 Da) alone in that of OSM. In addition, Bou-Mitri (2013) suggested that the fragment ions of the FA oligomers resulted from consecutive decarboxylations, demethylations and other fragmentations, where its oligomers formed in aqueous medium were linked via M_5-M_β binding by C-O coupling mode; hence concluding, that the FA dimers and trimers synthesized in SFM could be that of M_5-M_β type. Likewise, Carunchio *et al*. (2001) also reported the formation of fragmentation ions at *m/z* 282, 173, 159 and 123 after consecutive decarboxylations and demethoxylations of FA dimer (385 Da), during the ESI-MS analysis and indicated that these ions were characteristic of a M_5-M_β type.

Aljawish *et al*. (2014) characterized the laccase-catalyzed oxidation products of FA, using LC-MS/atmospheric pressure chemical ionization (APCI) in positive ion mode, where they indicated a dimer, with a MW of 387 Da; further analysis of the end product by MS^2 and MS^3 showed fragment ions that resulted from various decarboxylations, dehydrated parent ions and loss of CH. Similarly, Adelakun *et al*. (2012a) reported the LC-MS analysis of laccase-catalyzed end products of FA in OSM, using ESI-MS in the negative ion mode, where two dimers, P1 (385.11 Da) with

M_5-M_β and P2 (358.10 Da) with M_β-M_β linkages were obtained; these authors also indicated the presence of a trimer and tetramer, respectively, of MW 579.21 and 769.19 Da.

The overall experimental findings suggest that the end product fractions EP-F1, EP-F2, EP-F3 and EP-F5 consisted of oligomeric products of FA, including di-, tri- and tetramers, where a better insight of their chemical structure was provided by the MS analysis. In addition, the results indicate that the laccase-catalyzed coupling mode and the DP of FA are influenced by the nature of the reaction medium as well as by the immobilization matrix of the enzyme.

7.4.6. Determination of antioxidant capacity

Using DPPH and AAPH as the free radicals, the antioxidant capacity of FA and its synthesized end products was determined (Table 7.2). The experimental findings show that the DPPH half maximal inhibitory concentrations (IC_{50}) for the FA and laccase-catalyzed end products were, respectively, 0.19 and 0.17 mg/mL, with corresponding Trolox equivalent (TE) values of 7,8812 and 8,4326 µmol of TE/g ferulic acid. These results indicate that the laccase-catalyzed end products exhibited a relatively higher antioxidant capacity as compared to that of their initial substrate, FA.

Bou-Mitri (2013) reported that the dimer of FA, obtained by laccase-catalyzed oxidation in OSM, demonstrated a 2.7 and 14.5 fold higher ORAC value, respectively, as compared to that of FA as well as the one obtained in aqueous medium; this author suggested that the enhancement in the antioxidant capacity of the dimer could be due to the increase in the electron donating groups after their dimerization, which reduced the O-H bond dissociation energy and favored the resonance delocalization of the phenoxy radicals. Similarly, Adelakun *et al*. (2012a) indicated that the β-5 dimer, synthesized during the laccase-catalyzed oxidation of FA in OSM, demonstrated a higher DPPH scavenging and Trolox equivalent antioxidant capacity (TEAC) as compared to that of its substrate. In addition, Adelakun *et al*. (2012b) reported that the laccase-catalyzed 2,6-dimethoxyphenol dimer, obtained in sodium acetate/ethyl acetate medium, exhibited 119.32, 53.15, 93.25% enhancement in its antioxidant capacity for ferric reducing antioxidant power (FRAP), TEAC and DPPH, respectively, as compared to that of the substrate.

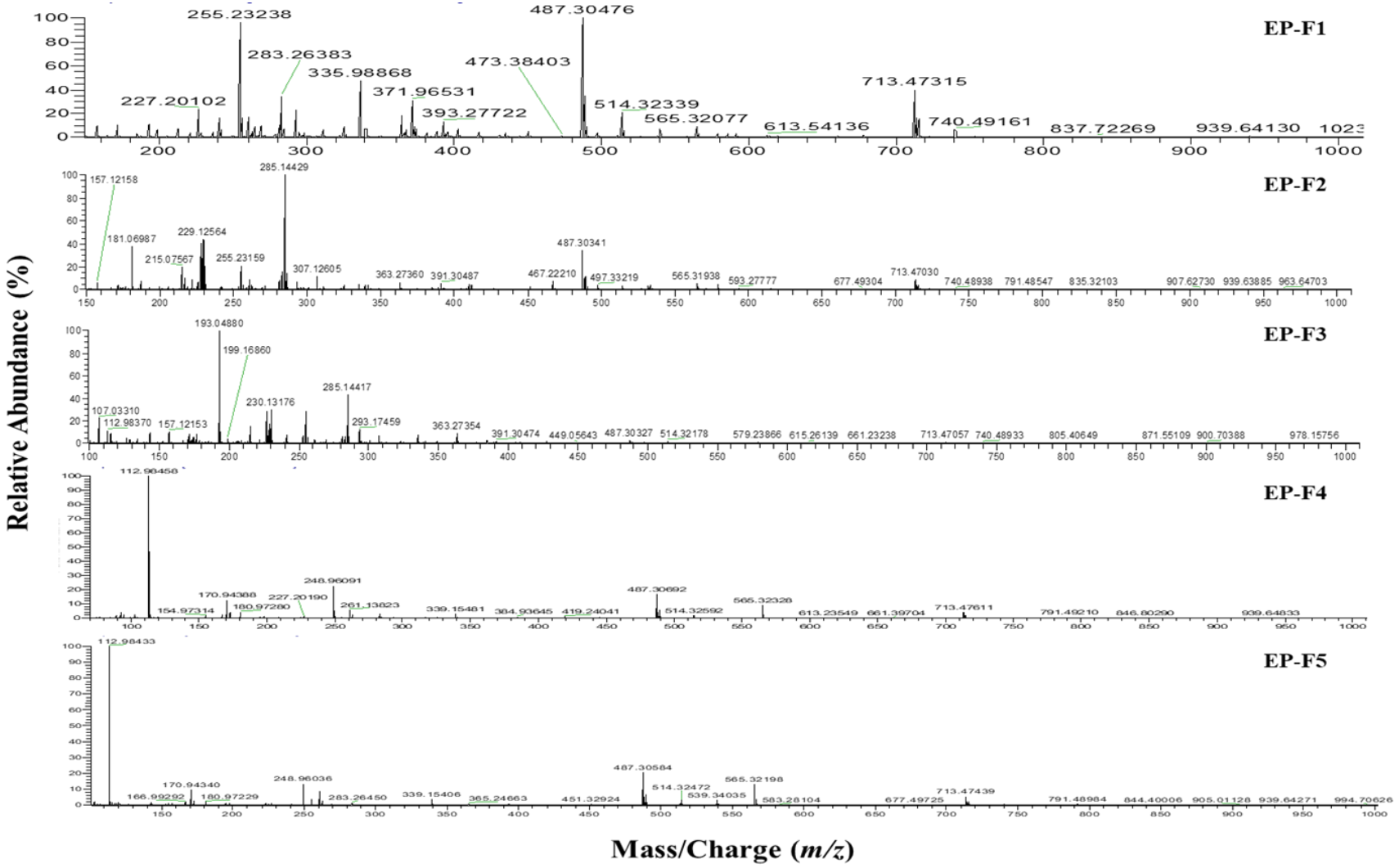


Figure 7.5. Electrospray ionization-mass spectrometry (ESI-MS) analysis of the laccase-catalyzed end product fractions, EP-F1, EP-F2, EP-F3, EP-F4 and EP-F5, using the LCQ Advantage, in the negative mode.

Table 7.2. Antioxidant capacity of ferulic acid and laccase-catalyzed end products in solvent-free media (SFM), using the oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay.

	Antioxidant capacity	
Products	Trolox equivalent (TE)[a]	IC_{50} DPPH (mg/mL)[b]
Ferulic acid	7,8812 (5.18)[c]	0.19 (6.15)
End product[d]	8,4326 (5.31)	0.17 (3.45)

[a]Trolox equivalents are expressed as μmol of TE/g of ferulic acid. All results are presented by means of triplicate trials.

[b]Half maximal inhibitory concentration (IC_{50}) is defined as the concentration (mg/mL) of the pure compound that results in 50% decrease in the DPPH concentration. All values are means of triplicate determinations.

[c]Relative standard deviation (RSD) was calculated by the standard deviation of triplicate trials divided by their mean, multiplied by 100.

[d]Laccase-catalyzed end product obtained in solvent-free media (SFM), composed of lyophilized ferulic acid and silica sol-gel encapsulated laccase enzymatic extract.

7.5. Conclusion

The present study suggests that the sol-gel encapsulated laccase-catalyzed oxidation of FA in SFM resulted in the synthesis of novel oligomeric structures that ranged from di-, tri- and tetramers with a relatively higher antioxidant capacity as compared to that of their initial monomer. The FA coupling mode and the extent of its oligomerization depends on the reaction medium as well as the method used for the immobilization of the laccase enzymatic extract. Overall, the results indicate that the sol-gel encapsulated laccase could be used as a potential biocatalyst in SFM for the production of natural oligophenols of higher antioxidant capacity.

GENERAL CONCLUSION AND CONTRIBUTIONS TO KNOWLEDGE

The overall objective of the present study aimed at optimizing the encapsulation of the laccase enzymatic extract, from *Coriolus hirsutus*, and its biocatalysis in solvent-free media (SFM) for the synthesis of selected oligophenols. The encapsulation of the laccase enzymatic extract in silica-sol gel matrix resulted in the enhancement of its catalytic activity and stability. A modification to the initial pre-gel alginate solution also led to an increase in the laccase activity, when encapsulated in a calcium alginate matrix. In addition, the hybridization of the alginate with silica appeared to be a promising approach for the encapsulation of a laccase enzymatic extract in organic polymers, where it improved the encapsulation efficiency (EE) of the matrix, as compared to that of the alginate alone.

Screening trials demonstrated the effects of selected silica sol-gel process parameters on the EE and the residual laccase activity of the encapsulated enzymatic extract. The use of longer gel aging and shorter gel drying times resulted in an increase in the laccase catalytic activity. The water to silane molar ratio (*r*), hydrochloric acid (HCl) content and protein loading appeared to be the most significant parameters in the sol-gel encapsulation process, where their optimization could provide an efficient method for the encapsulation of the laccase enzymatic extract in silica sol-gel.

A central composite rotatable design (CCRD) was successfully used to optimize the silica sol-gel encapsulation of our laccase enzymatic extract. Amongst the selected parameters, *r* was determined as the most significant independent variable and demonstrated an opposite interaction effect to that with the acid content, on the residual laccase activity. Using the optimum conditions, the encapsulated laccase enzymatic extract showed an enhanced residual laccase activity; although the sol-gel encapsulated laccase was characteristic of diffusional limitations, it also resulted in an increased catalytic efficiency. In addition, the encapsulated laccase enzymatic extract also demonstrated improved stability in terms of its pH, temperature, enzyme reusability and storage stability, as compared to that of the free one.

An effective method was also developed for the biocatalysis of the sol-gel encapsulated laccase enzymatic extract in SFM, using selected phenolic model substrates. With ferulic acid (FA) as a substrate model, the sol-gel encapsulated enzymatic extract successfully carried out its biocatalysis in SFM, with a significant enhancement in the laccase activity as compared to that obtained in the

aqueous medium. In contrast, under the same conditions, no catalytic activity was obtained for the free laccase enzymatic extract in SFM. The experimental findings indicated that the encapsulation of the laccase enzymatic extract not only led to an enhancement in its catalytic activity, but could also promote its application as a potential biocatalyst in such non-conventional reaction medium.

Using FA as a substrate model, structural analyses for the sol-gel encapsulated laccase-catalyzed end products were also carried out. The results showed that the laccase-catalyzed oxidation of FA in SFM, led to the formation of five end product fractions which comprised of a mixture of di-, tri- and tetramers with C-O-C and/or C-C linkages. In addition, the synthesized FA oligomers demonstrated a relatively higher antioxidant capacity as compared to the intial monomer.

Contributions to knowledge:

The overall study resulted in a successful use of sol-gel encapsulated laccase enzymatic extracts, for the solvent-free synthesis of FA oligomers of high antioxidant capacity. The optimization of the sol-gel encapsulation method for the laccase enzymatic extract could broaden its use as a potential biocatalyst with enhanced enzyme stability, and may contribute to the development of various industrial bio-processes. In addition, the research work succeeded in the development of a novel method for carrying out the laccase-catalyzed biocatalysis in SFM, with the use of the sol-gel encapsulated laccase and lyophilized substrate. The use of such reaction media is an appropriate choice in green chemistry, as an approach for lowering the environmental and economical impacts of biochemical processes. Overall, the encapsulation of laccase enzymatic extract in silica sol-gel as well as its biocatalysis in SFM could lay the ground for a biotechnological approach, aimed at the biosynthesis of oligophenols of relatively higher antioxidant capacity, for their application in the development of food additives, nutraceuticals or health products.

Recommendations for future research:

Future research work should be aimed at the development of a scale-up method for the encapsulation of laccase in silica sol-gel. In addition, research should be directed towards the development of production as well as the scale-up for the oligophenol end products. Once this is achieved, the nutritional and safety quality of the end products should also be assessed.

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