FERMENTATION AND KINETIC STUDIES ON LACCASE PRODUCTION BY *PYCNOPORUS SANGUINEUS*

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ABSTRACT

Laccase (benzenediol: oxygen oxireductase, EC 1.10.3.2) is an extracellular enzyme that belongs to the blue multi-copper oxidases group. The broad specificity enzyme has the ability to oxidize wide range of aromatic compound especially phenolic compounds through radical-catalyzed mechanism involving four electrons reduction of oxygen molecule into water. Hence, laccase has been used in many biotechnological applications especially in pulp and paper industry, wastewater treatment, bioremediation, biosensor, etc.

Laccase is mostly produced by white rot fungi which belong to the basidiomycetes. In this study, laccase was produced by a white rot fungus *Pycnoporus sanguineus* using submerged fermentation. Medium improvement for laccase production using shake flasks, the effects of yeast extract, malt extract and peptone were tested using two level full factorial design. The effect of malt extract was significant in improving extracellular laccase production. Different initial glucose concentrations tested did not significantly improved laccase production in the shake flasks after seven days cultivation.

The effects of agitation speed and flask type on laccase production in shake flask was studied. Highest laccase productivity was obtained when the fungus was cultivated in the baffled flasks at 170 rpm. However the used of baffled flasks at higher agitation speeds resulted in excessive cell growth on the wall which decreased the enzyme production.

In the production of laccase using stirred tank reactor (STR) selected culture variables were optimized using response surface methodology. The optimization process was performed in two stages viz screening experiment and followed by the optimization. The effects of selected operating variables namely

agitation rate (rpm), aeration rate (L min^{-1}) and pH were screened using two level full factorial design. Agitation rate and pH which significantly influenced laccase production in the screening experiment were furthered optimize using face-centered central composite design (FCCCD). Based on the FCCCD experiments, the predicted maximum laccase activity was 67.2 U L^{-1}. Validation experiments using optimized conditions (agitation 600 rpm, pH 4.43 and aeration 1.0 L min^{-1}) yielded average laccase production of 62.9 ± 4.9 U L^{-1}.

The kinetics of laccase production in shake-flask and stirred tank reactor were successfully modelled using unstructured kinetic model. The differential equation was solved using non linear regression method in order to obtain the kinetic parameters for biomass (*X*), laccase (*P*) and glucose content (*S*). Simulated model and experimental data showed good agreement. Based on the kinetic studies, laccase production increases with the depletion of glucose and when biomass growth has reached stationary phase. Thus, the laccase production in this fungal strain can be classified as non growth related.

Partial purification of laccase was performed in three steps including ammonium sulfate precipitation, desalting and protein fractionation. Packed column was used in gel chromatography studies with Sephadex G-25 and G-75 as gel matrix for the column separation in desalting and protein fractionation, respectively. The purification fold value for the ammonium precipitation, Sephadex G-25 and Sephadex G-75 were 1.1, 3.8 and 5.1, respectively. Based on SDS PAGE analysis, the protein bands from the purified sample were located within 66 to 97 kDa range.

ABSTRAK

‘Laccase’ (benzenadiol; oksigen oksireduktase; EC 1.10.3.2) adalah enzim luar sel tergolong di dalam kumpulan muli-kuprum oksidase. Ketentuan enzim yang luas di dalam pengoksidaan sebatian aromatik melalui tindak balas bermangkin radikal melibatkan penurunan oksigen kepada air dengan kehadiran empat elektron. Enzim ‘laccase’ telah digunakan di dalam pelbagai aplikasi bioteknologi seperti industri penghasilan kertas dari pulpa, rawatan air tercemar, bioremediasi, biopenderia dan sebagainya.

Kebanyakan enzim ‘laccase’ dihasilkan oleh kulat kulapuk putih yang tergolong di dalam kumpulan basidiomiset. Melalui kajian ini, ‘laccase’ telah dihasilkan oleh kulat kulapuk putih *Pycnoporus sanguineus* dengan menggunakan teknik penapaian tenggelam. Di dalam kajian peningkatan media penghasilan ‘laccase’ di dalam kelalang goncang, pengaruh ekstrak yis, ekstrak malt dan pepton telah dikaji menggunakan reka bentuk faktoran penuh. Kajian mendapati pengaruh ekstrak malt menunjukkan kesan yang ketara di dalam peningkatan penghasilan ‘laccase’ luar sel. Kajian penggunaan kepekatan awal glukosa yang berbeza tidak memberi kesan yang ketara di dalam peningkatan penghasilan ‘laccase’ luar sel dengan menggunakan kelalang goncang selepas 7 hari penuaian.

Kesan kelajuan goncangan dan jenis kelalang goncang terhadap penghasilan ‘laccase’ telah dikaji. Penghasilan ‘laccase’ tertinggi diperolehi daripada kulat yang ditumbuhkan di dalam kelalang jenis sesekat pada kelajuan goncangan 170 rpm. Penggunaan kelalang sesekat pada kelajuan goncangan tertinggi telah menyebabkan pertumbuhan di permukaan dinding meningkat dan seterusnya menurunkan penghasilan ‘laccase’.

Di dalam kajian penghasilan ‘laccase’ menggunakan reaktor tangki pengaduk, pemboleh ubah kultur terpilih telah dioptimumkan menggunakan kaedah permukaan gerak balas. Melalui kaedah gerak balas permukaan, proses yang terlibat adalah terbahagi kepada dua peringkat iaitu eksperimen saringan diikuti dengan proses pengoptimum. Kesan kelajuan pengaduk (rpm), kadar pengudaraan (L min^{-1}) dan pH telah diuji saring menggunakan reka bentuk faktoran penuh dua peringkat. Kesan kelajuan pengaduk dan pH yang ketara telah digunakan di dalam proses pengoptimum menggunakan reka bentuk respons permukaan berpusat muka. Berdasarkan eksperimen respons permukaan berpusat muka, jangkaan penghasilan ‘laccase’ yang maksimum adalah 67.2 U L^{-1}. Eksperimen pengesahsahihan dengan menggunakan keadaan yang optimum (kelajuan pengaduk 600 rpm, pH 4.43 dan kadar pengudaraan 1.0 L/min) telah menunjukkan penghasilan ‘laccase’ sebanyak 62.9 ± 4.9 U L^{-1}.

Kinetik penghasilan ‘laccase’ menggunakan kelalang goncangan dan reaktor tangki pengaduk telah berjaya dengan dimodelkan menggunakan model kinetik tanpa struktur. Persamaan pembezaan diselesaikan dengan menggunakan kaedah regresi tak linear untuk mendapatkan parameter kinetik bagi biojisim (X), ‘laccase’ (P) dan kandungan glukosa (S). Simulasi model dan data eksperimen bertepatan antara satu sama lain. Berdasarkan kajian kinetik, penghasilan ‘laccase’ meningkat dengan penurunan glukosa dan ketika biojisim memasuki fasa pegun. Maka ‘laccase’ yang terhasil daripada strain kulat yang digunakan tergolong di dalam kumpulan yang tidak berkait rapat dengan pertumbuhan.

Proses penulenan separa telah dijalankan melalui tiga peringkat melibatkan pemendakan ammonium sulfat, penyahgaraman dan pemeringkatan. Dengan menggunakan turus terpadat, matriks gel Sephadex G-25 dan G-75 masing-masing telah digunakan sebagai matriks di dalam turus penyahgaraman dan

pemeringkatan. Nilai gandaan penulenan bagi pemendakan ammonium, Sephadex G25 dan Sephadex G-75 masing-masing adalah 1.1, 3.8 dan 5.1. Berdasarkan analisis ‘SDS PAGE’, jalur-jalur protein terhasil daripada sampel penulenan terletak di dalam julat lingkungan 66 hingga 97 kDa.

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LIST OF ABBREVIATIONS

KH_2PO_4	Ammonium sulfate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
CaCl	Calcium chloride
KCl	Potassium chloride
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
FFD	Full factorial design
ANOVA	Analysis of variance
FCCCD	Face-centred central composite design
CCD	Central composite design
PES	Polyethersulfone
STR	Stirred tank reactor
pK_a	Acid dissociation constants
$[A^-]$	Conjugate base proportion
$[HA]$	Acid proportion
DNS	Dinitrosalicylic acid
BSA	Bovine serum albumin
$\Delta A/\Delta t$	Absorbance changes per minute (min^{-1})
ε	Syringaldazine molar extinction coefficient (65000 M^{-1} cm^{-1})
l	Light path length (cm)
v_t	Total assay volume (L)
v_s	Total enzyme sample (L)
X	Biomass production (g L^{-1})
S	Glucose utilization (g L^{-1})
P	Laccase production (U L^{-1})
ln	Natural logarithm
dX/dt	Rate of biomass production (g L^{-1} day^{-1})
U_{max}	Maximum specific growth rate (day^{-1})
X_{max}	Maximum biomass concentration (g L^{-1})
t_{Xmax}	Cultivation time for maximum biomass concentration (day)
LP	Luedeking-Piret
dP/dt	Rate of laccase production (U L^{-1} day^{-1})
α	Coefficients for growth associated product (U g^{-1})
β	Coefficient for non-growth associated product (U g^{-1} day^{-1})
dS/dt	Rate of utilization of glucose (g L^{-1} day^{-1}),
Y_G	Coefficient of biomass yield over substrate (g g^{-1})
Y_{PS}	Coefficient of product yield over substrate (g g^{-1}),
m_S	Cell maintenance coefficient (g g^{-1} day^{-1})
G_{sat}	Amount of salt contained in 1 L of 100% saturated solution (g)
S_1	Initial concentration of saturated solution (%)
S_2	Concentration of saturated solution (%)
P_{Salt}	Salt saturation constant

V_T	Total column volume (mL)
V_0	Void volume (mL)
V_M	Gel matrix volume (mL)
V_E	Total volume of eluted sample (mL)
V_{SL}	Total volume of sample loaded (mL)
R^2	Correlation coefficient
R^2 adjusted	Adjusted correlation coefficient
S_{Error}	Standard error regression
PRESS	Prediction sum of squares
Coef	Coefficient
σ	Standard deviation
SEM	Standard error mean
SE	Standard error
N	No of sample
DoF	Degree of freedom
SS	Sum of squares
MS	Mean on squares
Adj SS	Adjusted sum of squares
Adj MS	Adjusted mean of squares
F	F statistic
P	P statistic
T	T statistic
r_i	Actual residual observation
d_i	Standardized residuals
y_i	Response observation

CHAPTER ONE

1.0 INTRODUCTION

Biotechnology is a multidisciplinary field of research which require basic scientific knowledge such as microbiology, biochemistry, physics, chemistry and engineering disciplines including chemical and bioprocess engineering. The application of biotechnology in the industry activity including food, animal feedstock, environment, healthcare, agriculture and energy had improved people social life and economy. Industrial biotechnology is the application of biological based system and their components in manufacturing industrial products or as tool for industrial processes.

Industrial biotechnology is also known as “White biotechnology”. The advantages of using biotechnology in the industry are low process energy, reduce pollution and generate biodegradable waste which more environmental friendly. Biotechnology can be a tool for solving certain problems which conventional process has failed to deliver. Some of the conventional methods are less efficient in terms of cost and time. Certain conventional process also produce by-product which sometime could be harmful to the environment. In some developed country, industrial biotechnology is required to support the needs of their population. Food supply, energy source and healthcare are the most important aspect in this modern world. Only using industrial biotechnology, those aspects can be fulfilled with respect to sustainable development.

Enzyme is one of the most promising products from industrial biotechnology. In many biocatalyst processes, enzyme acts as a catalyst to speed up specific chemical reactions. Enzyme is made from sequence of amino acids with different three-dimensional structures which gives the molecule unique

properties. Several enzymes have broad substrate specificity which has been exploited in several biotechnological applications. In the current days, modernization has resulted in more problems related to the environmental problems. Large number of pollutants keeps increasing as a result of industrial activities. The used of enzyme in solving environmental problems was due to costs effectiveness which are characterized by higher catalytic ability, wide range of substrate, less toxicity, mild reaction conditions, etc (Alcalde *et al.*, 2006).

Since nineteenth century, laccase has been the subject of study and interest in biotechnology mainly due their ability to oxidise a wide range of aromatic compounds and non-aromatic compounds by a radical-catalysed reaction mechanism. From previous studies, laccase has been found to degrade phenolic compound. The wide range of substrate specificity and strong oxidative activity has made laccase enzyme become more important compare to other oxidative enzymes such as lignin peroxidise (LiP) and manganese peroxidise (MnP). Due to its numerous advantages, the mass production of the enzyme in order to fulfil the needs of industrial sector is one of the main thrust for many biotechnological companies. One way to produce laccase is through the fermentation process using microorganism such as white rot fungi.

Renewable and environmental friendly materials have been used as nutrients and substrate during enzyme production. One of the limiting factors that influence the large-scale fungal laccase productions is the lack of efficient system at production scale. Optimization of fermentation parameters is required for maximum laccase productivity. This can be archived by using statistical experimental design which can provide us the knowledge and better explanation of important parameters involved in the process.

The main objectives of the study are:

i. To optimize the production of laccase using statistical experimental design in stirred tank reactor;

ii. To study the effect of different cultivation parameters on fungal growth, laccase production and glucose utilization;

iii. To model the production of laccase, fungal growth and glucose utilization in shake flasks and stirred tank reactor;

iv. To partially purify laccase from the fermentation process to an acceptable level.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Historical Background

Laccase (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) is a multicopper enzyme that belongs to blue oxidases group. Catalytic activity of this enzyme includes a wide range of substrates such as phenolic compounds, aromatic amines, diamines, etc., The reactions accompanied by the reduction of two oxygen molecules to water. Laccase was first detected in the studies on sap of Japanese lacquer tree *Rhus vernicifera* by Yoshida in 1883. According to Thurston (1994), laccase production from fungal species was first demonstrated by Betrand and Laborde in 1896. Laccase has been found in a variety of fungi including yeasts (e.g. *Cryptococcus*), molds (e.g. *Penicillium*), and mushrooms (e.g. *Agaricus*). Laccase also have been discovered in bacteria which is restricted to a few species from genera *Azospirillum* (Alexandre and Bally, 1999), *Sinorhizobium* (Castro-Sowinski *et al.*, 2002), *Streptomysces* (Endo *et al.*, 2002) and *Bacillus* (Martins *et al.*, 2002). The presence enzyme with features of typical laccases have also been reported in insects (Kramer *et al.*, 2001).

2.2 Laccase from *Pycnoporus sanguineus*

It has been reported that laccase is widely produced by white rot fungi species which is belonging to wood decay fungi (Mayer and Staples, 2002; Youn *et al.*, 2006; Fonseca *et al.*, 2010; Hildén *et al.*, 2012). The main characteristic of this organism is that it has the ability to grow on decomposed wood which usually turns whitish and slightly brown with fibrous appearance. The colour is due to the bleaching of wood by the phenoloxidase activity which responsible in lignin

oxidation. Most of white-rot fungi produce three types of phenoloxidase which are lignin peroxidase (LiP); manganese peroxidase (MnP) and laccase (Lac) (Arora *et al.*, 2002; Wesenberg *et al.*, 2003; Fujii *et al.*, 2013; Janusz *et al.*, 2013). Laccase is particularly abundant in many white rot fungi and mainly involved lignin metabolism by the organism (Bourbonnais *et al.*, 1995; Leontievsky *et al.*, 1997). Despite that, there are also accessory enzymes such as H_2O_2-forming glyoxal oxidase (GLOX), aryl alcohol oxidase (AAO), oxalate producing oxalate decarboxylase (ODC), NAD-dependent formate dehydrogenase (FDH) and P450 monooxygenase that have been isolated from many white-rot fungi strains (Wesenberg *et al.*, 2003; Aguiar *et al.*, 2006).

According to Smânia *et al.* (1995), white rot fungus, *Pycnoporus sanguineus* is classified as Basidiomycetes of *Polypraceae* family. Due to their ability of degrading lignin, *P. sanguineus* can easily grow on solid wood by producing an enzyme (Heerden *et al.*, 2008) which is known as lignin modifying enzyme (Cavallazzi *et al.*, 2004). At 30 °C, lignin degradation by *P. sanguineus* was higher compared with degradation at 25 °C. Smânia *et al.* (1998) has indicated that the orange colour of *P. sanguineus* is due to the cinnabarin effect which is an orange pigment.

Antimicrobial studies have indicated that *P.sanguineus* has antimicrobial effect on other organisms. The culture broth was shown to contain antibacterial activity against strains of *Escherichia coli*, *Klebsiella penumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aereus* and the members of the genus *Streptococcus* (Smânia *et al.*, 1995). The culture broth was also sigificantly more active on gram-positive cocci than on gram-negative bacilli (Smânia *et al.*, 1995). The fungal has play significant role in the removal of dangerous compound from the environment such as metals, phenolic compounds, textile dyes and etc.

Zulfadhly *et al.* (2001) have investigated the ability of *P. sanguineus* to absorb heavy metals from aqueous solution in fixed bed column reactor where they found that fungus biomass was an effective biosorbent for the removal of Pb^{2+}, Cu^{2+} and Cd^{2+} metals. A mass transfer model has been proposed for the removal of lead, copper and cadmium using *P. sanguineus* as biosorbent (Yus Azila *et al.*, 2008; Yahaya *et al.*, 2009).

2.3 Characterization and Properties of Laccase

2.3.1 Structure

The main features fungal laccase structures are generally glycosylated with glycosylation ranged of 10 to 30 % and only few cases higher than 30 % (Shleev *et al.*, 2004). According to Rodgers *et al.* (2010), protein glycosylation are involved in the stabilization of catalytic centre against hydrolysis and protection from thermodegradation of the enzyme. Laccase is a multi-copper enzyme which contained at least four types of coppers (T1, T2, T3 and T3'). T1 copper site is a primary oxidation site which accepts electron from substrate through Cys-His pathway. The electrons transfer into the tri-nuclear site (T2, T3 and T3') at which oxygen molecule is reduced to water (Piontek *et al.*, 2002; Claus, 2004).

The spectroscopic behaviour of all four copper sites shows different maximum adsorption. T1 site has maximum adsorption at 600 nm also known as paramagnetic blue copper. Spectroscopic studies on T3 and T3' copper sites indicated maximum adsorption at 310 nm. The T2 copper shows only weak adsorptions in the visible region. The T2 copper cannot be detected using spectrophotometer studies; however, it generates a characteristic electron paramagnetic resonance (EPR) signal (Leontievsky *et al.*, 1997). Catalytic efficiency of laccase for some substrates depends linearly with redox potential of

laccase. T1 site possessed high redox potential which is special interest for biotechnological applications.

Ducros *et al.* (1998) reported the crystal structure of a Type-2 depleted form of laccase from *Coprinus cinereus*. The putative T2 copper is completely absent and catalytic activity is in the incompetent state. Investigation on complete laccase structure with four coppers have been carried out in *Trametes versicolor* (Bertrand *et al.*, 2002; Piontek *et al.*, 2002), *Melanocarpus albomyces* (Hakulinen *et al.*, 2002), *Bacillus subtilis* (Enguita *et al.*, 2004) and *Rigidoporus lignosus* (Garavaglia *et al.*, 2004). Crystallization of laccase indicates excellent electron density of the molecule which later can be used for modelling of laccase structure. However, high quality crystallization can cause deglycosylation of enzyme which resulted in the loss of copper. Antorini *et al.* (2002) had purified laccase isoenzymes from *Trametes versicolor* and *Pycnoporus cinnabarinus* to apparent isoelectric homogeneity without glycosylation. These proteins samples were fully active with high resolution of crystal structure (Antorini *et al.*, 2002). Recently, a complete crystal structure of laccase in oxidized form with complete 4 copper states has been discovered (Piontek *et al.*, 2002). The overall crystal structure of *T. versicolor* laccase indicates 7 N-acetyl glucosamine moieties at 5 distinct N-glycosylation sites.

According to Piontek *et al.* (2002), a monomer structure of laccase from *T. versicolor* has dimensions of about 65 x 55 x 45 Å which is represented by three domains i.e. domain 1 (D1), domain 2 (D2) and domain 3 (D3). Domain 1 (D1) contain copper site 1 (T1) whereas domain 2 (D2) and 3 (D3) consists of tri-nuclear copper site. The terminal end of domain 3 is made of carbon group. The electrostatic surface potential of the crystal structure reveals dominance

distribution of negative charge with acidic pI value of 3.5 (Piontek *et al.*, 2002). Laccase structure suggested by Piontek *et al.* (2002) is showed in Figure 2.1.

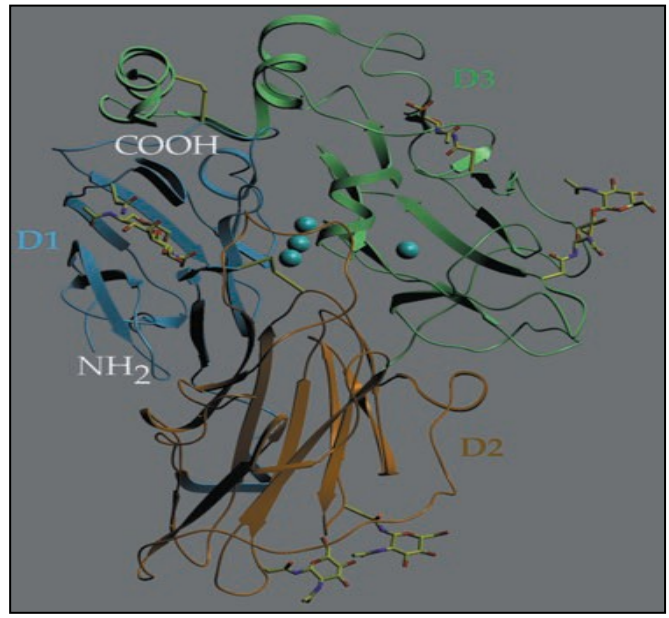


Figure 2.1: Laccase structure from *T. versicolor* (Piontek *et al.,* 2002)

2.3.2 Molecular Weight

Laccase from more than 30 species of fungi have been purified and characterized and primary structures of many have been determined by protein or DNA sequencing (Ong *et al.*, 1997; Temp and Eggert, 1999; Dedeyan *et al.*, 2000). Laccase vary considerably in size depending on method of size estimation such as electrophoresis, size exclusion, ultracentrifugation and predicted size from gene sequence. Laccase exhibit a molecular mass of 50 to 90 kDa in SDS-PAGE. The majority of the fungal extracellular laccases are approximately 60 to 70 kDa with an acidic isoelectric point (p*I*) around pH 4.0 (Shleev *et al.*, 2004).

2.3.3 Substrates and Inhibitors

Numerous substrates have been used in the study of laccase activity. According Mayer and Staples (2002), laccase is able to oxidize quinol as

determined by oxygen uptake no matter what others substrate are attacked. The oxidation of a substrate by laccase typically involves the loss of a single electron and the generation of free radical (Solomon *et al.*, 1996). The one electron oxidation of hydroxylated aromatic substrates is accompanied by the reduction of molecular oxygen to water by the transfer of four electrons. The nature and position of substituent in the phenolic ring influences the efficiency of oxidation by a particular laccase. Natural substrates such as plant cell wall materials can be depolymerized by laccase through enzyme-substrate oxidation. Table 2.1 showed several substrates for laccase activity determination.

Table 2.1: Substrates for laccase enzyme assay

Substrate	Reaction condition		References
	Buffer	pH	
ABTS, 2,2-azinobis (3-ethylbenzthiazoline-6-sulfonate)	Sodium succinate 25 mM acetate. 100 mM phosphate	4.5 3.8 5.7	(Virtanen *et al.*, 2012) (Songulashvili *et al.*, 2007) (Klonowska *et al.*, 2002)
Syringaldazine	50 mM citrate. 50 mM phosphate. 50 mM acetate.	4.8 6.0 6.0	(Garcia *et al.*, 2006) (Medeiros *et al.*, 1999) (Grover *et al.*, 2012)
DMP, (2,6-dimethoxyphenol)	100 mM glycine-NaOH 100 mM sodium-phosphate.	10.0 5.9	(Gali and Kotteazeth, 2012) (Claus and Filip, 1997)
Guaiacol	50 M succinic acid-NaOH.	4.5	(Xiao *et al.*, 2003)
Catechol	100 mM citrate-phosphate.	4.5	(Shleev *et al.*, 2007)
Hydroquinone	100 mM sodium-acetate	4.5	(Chakroun *et al.*, 2010)

However precautions are needed since syringaldazine is also oxidized by manganese peroxidase and lignin peroxidase which might be present in crude mixture. If the reaction mixture contains hydrogen peroxide, syringaldazine can

also be oxidized by manganese peroxidase and lignin peroxidase. The used of catalase to convert hydrogen peroxide into water and oxygen molecule is one of the way to confirm the reaction is solely due to laccase enzyme (Lonergan and Baker, 1995).

Enzyme inhibitors are molecules that interact with the enzyme to prevent it from working in the normal manner. There are a variety of types of inhibitors including; nonspecific, irreversible, reversible-competitive and non-competitive. The effect of inhibitor on laccase catalytic mechanism has been widely study. The effect of hydroxylamine and sodium azide on laccase will result changes in kinetic analysis of the enzyme. When using hydroxylamine as inhibitor, K_m value of laccase with ABTS substrate was increased. The inhibitor constant (K_i) of hydroxylamine is 1.33 mM and it was shown that hydroxylamine act as competitive inhibitor (Mishra and Kumar, 2009). On addition of non competitive inhibitor such as sodium azide, V_{max} was significantly decreased and K_i value for sodium azide was determined to be 0.1826 mM (Mishra and Kumar, 2009). According to previous study by Johannes and Majcherczyk (2000), sodium azide completely stopped the oxygen uptake for laccase-catalysed oxidation of ABTS when added into the enzyme reaction mixture.

2.3.4 Temperature and pH

The active sites of laccase enzyme are composed of ionizable groups which should in the proper ionic form in order to maintain their catalytic ability. The optimum pH for *P.sanguineus* laccase enzyme activity using DMP and guaiocol was 4 (Litthauer *et al.*, 2007). Laccase activity was observed high in acidic region (pH 3) when using ABTS as substrate (Litthauer *et al.*, 2007). If the reaction takes place outside the optimal pH, the enzyme activity is decreased. The changes of pH

also affect the stability of laccase enzyme. Like most enzyme reactions, the rate of reaction increases as the temperature is raised. However, high temperature may cause the denaturation of laccase enzyme. Temperature effect on laccase enzyme is important. Recent studies showed half life ($t_{1/2}$) of laccase enzyme from white rot fungus *Marasmius quercophilus* was found to be 74 minutes at relatively higher temperature of 60 °C with optimum temperature for laccase activity was observed at 75°C (Dedeyan *et al.*, 2000).

2.3.5 Laccase Reaction Mechanisms

Laccase reaction mechanism is a process where substrates are being oxidized with the concomitant reduction of molecular oxygen to water. Although there are many enzymes that utilize oxygen molecule, only a few reduce it completely to water. The enzyme reacts with wide range of substrates. Substrate oxidation by laccase is a one-electron reaction that generates a free radical. Catalytic cycle of laccase (with 4 copper atoms) shows the reduction of one oxygen molecules into two water molecules (Yaropolov *et al.*, 1994; Shleev *et al.*, 2006).

According to Solomon *et al.* (2008), mechanism of oxygen molecule reduction to water involved the formation of intermediates; peroxy intermediate (PI) and native intermediate (NI). Overall reaction proceeds *via* two sequential steps. The first step is oxidation of substrate which T1 site will accept four electrons from the oxidation of oxygen molecule. During the process, radical species, PI is generated. The oxygen bond in PI structure will undergo reductive cleavage. The second step is a molecule rearrangement of PI which NI will be generated. The PI is a two e^- reduced species and NI is four e^- reduced species.

Finally, NI will be oxidized completely by the formation of two water (H_2O) molecules.

Laccase substrates can be divided into phenolic and non-phenolic compounds. Phenolic substrates such as syringaldazine, dimethoxyphenol (DMP), guaiocol and electron donor substrates such as ABTS and ferrocyanide (Yaropolov *et al.*, 1994; Jarosz-Wilkołazka *et al.*, 2005) are commonly used. The oxidation of phenolic substrates to phenoxy radicals depends on reaction condition. Phenolic substrates will be oxidized *via* (i) radical coupling or (ii) molecule rearrangement to quinones which overall reactions involve alkyl-aryl cleavage, C_α oxidation, cleavage of C_α–C_β bond and aromatic ring cleavage (Kawai *et al.*, 1988).

Oxidation of non-phenolic and high redox potential substrate by laccase requires a mediator. Most of the mediators are easily oxidized and progressively removed from the reaction medium undergoing polymerization except in the case TEMPO and its cognates (Astolfi *et al.*, 2005). In laccase mediator system (LMS), laccase indirectly oxidize the non phenolic substrate with mediator by extending laccase reactivity. ABTS has been used as the first mediator in laccase-mediator system (LMS) for pulp delignification (Bourbonnais and Paice, 1992). Compound with high oxidation potential with long half life enough to permit the diffusion towards substrate is suitable for an effective redox mediator. The effect of mediator in the substrate oxidation is presented in Figure 2.2.

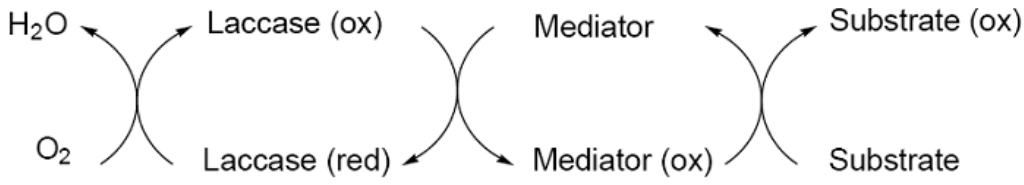


Figure 2.2: Role of laccase mediator (Baiocco *et al.,* 2003)

Laccase oxidations of non-phenolic substrates proceeds *via* two mechanisms which are electron transfer (ET) route and hydrogen atom transfer (HAT) route. These mechanisms depend on specific mediator such as ABTS and HBT. In the case of HBT type mediator, nitroxyl radical (N-O) of HBT is oxidized through hydrogen atom transfer route (HAT route). Product from this oxidation is in the form of ketone. In the ET route, non-phenolic substrate will oxidize ABTS into radical ($ABTS^{+}$ or $ABTS^{2+}$) and produce aldehyde as oxidation product. The oxidation of non-phenolic substrate is described in Figure 2.3.

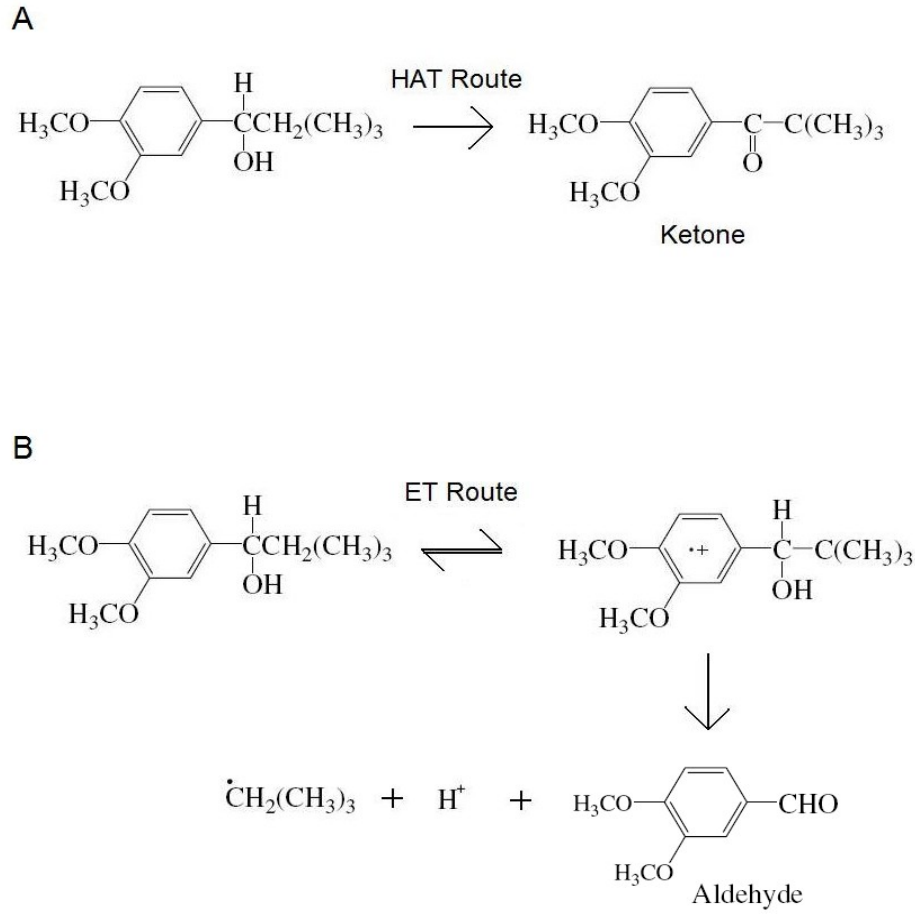


Figure 2.3: Laccase mediated oxidation of non-phenolic substrates via
A: Hydrogen Atom Transfer Route (HAT Route) and B: Electron Transfer Route (ET Route) (adapted from Galli and Gentili (2004))

2.4 Industrial Application of Laccase

Due to its ability to oxidize various compounds at the expense of molecular oxygen, laccase has been used in various biotechnological applications. Compared to the conventional process, the reaction mechanism involving laccase exhibit more environmental friendly process with water as the only by-product. The used of laccase as biocatalyst in many conventional process which mainly using chemical process has significantly reduced the production cost. Furthermore the appearance of non-desired product due to the side reaction mechanisms from the conventional process can be avoided by using laccase as the enzyme has wide range of substrate.

2.4.1 Lignin Degradation in Pulp and Paper Industry

Lignin is one of the main components of wood and resistant to hydrolytic breakdown. In lignin degradation, phenoloxidase laccase is involved. Laccase provide advantages such as greater stability and wide range of substrates. The ability of laccase enzyme in the removal of lignin compound has attracted many researches due to its importance in the pulp and paper industry. Conventional methods utilize chlorine-based agents which can lead to the release of toxic contaminants to the environment. Conventional methods using chlorine will generate by-product of chlorinated organic compounds which is very toxic to the environment. Environmental friendly bleaching system using enzyme through oxidative delignification process has been industrially introduced (Pereira *et al.*, 2005; Tian *et al.*, 2012).

The process of using laccase enzyme in the delignification is called laccase mediator system (LMS). The potential of LMS to remove lignin-derived products responsible for colour from a high quality flux pulp have been reported (Camarero

et al., 2007; Fillat and Roncero, 2009). Laccase also can be used in the enzymatic adhesion of fibres in the manufacturing of lignocelluloses-based composite materials such as fibreboards without using toxic synthetic adhesives (Felby *et al.*, 2004; Álvarez *et al.*, 2011).

2.4.2 Industrial Wastewater Treatment

Biotechnological treatment of industrial effluents generated by printing and dye industry with laccase requires a large amount of enzyme. Treatment of the effluent with enzymes has attracted great attention in recent years (Daâssi *et al.*, 2012; Tišma *et al.*, 2012). Recently laccase from several microbial sources has been employed for the decolorization of various categories of dyes such as triphenylmethane, azo, indigoid, anthraquinone and heterocyclic/polymeric dyes (Couto and Herrera, 2006; Hadibarata *et al.*, 2012). The enzyme also able to decolorize reactive dyes such as Remazol Brilliant Blue R (Soares *et al.*, 2001) and Remazol black-5 (Murugesan *et al.*, 2007). Decolorization of synthetic dyes by *Trametes modesta* most efficiently occur under acid conditions (pH 3-6) with the rate of decolorization increase with temperature from 50 to 60 °C (Nyanhongo *et al.*, 2002). Laccase-based decolorization are potentially advantageous to the treatment of dye pollution since the enzyme can be produced in large amount and require less fastidious induction than either lignin peroxidase (LiP) or manganese peroxidase (MnP) (Pointing *et al.*, 2000).

Removal of phenolic or xenobiotic pollutants from wastewater using laccase can be accomplished in several ways. Ryan *et al.* (2005) has studied the removal of phenolic effluents using airlift reactor with maximum removal of phenol over biomass per day achieved at 0.033 $g_{Phenol}\ g^{-1}_{Biomass}\ day^{-1}$. It was reported that some phenols can inhibit the growth of white rot fungi (Buswell and

Eriksson, 1994). However adverse effects might be minimized by choosing appropriate dilutions and attaining appropriate stages of fungal growth. Using the appropriate flow rate of wastewater for remediation was shown to be important in experiments with dye-laden effluent (Romero-González *et al.*, 2006), where higher or lower rates led to laccase deactivation or inefficient system operation, respectively.

Wastewater treatment with laccase producing fungi growing directly is a relatively recent idea. The application of growing fungi on inert materials prior to exposure of the latter to effluent is one of the possibilities that have been tested in several studies (Rodríguez Couto *et al.*, 2004). Most experiments on the bioremediation of wastewater have been carried out with sterilized effluent. Nevertheless, Pedroza *et al.* (2007) successfully demonstrated that *Trametes versicolor* can treat wastewater from the pulp and paper industry under non-sterile conditions.

2.4.3 Bioremediation

Fungal enzymes are useful for the degradation of environmental pollutants. Many of the enzymes responsible for pollutant degradation are extracellular. In nature, most of the enzymes probably are involved in the degradation of wood. Laccase has also shown to be useful for the removal of toxic compounds through oxidative enzymatic coupling of the contaminants, leading to insoluble complex structures (Wang *et al.*, 2002). Phenolic compounds are present in wastes from several industrial processes, as coal conversion, petroleum refining, production of organic chemicals and olive oil production among others (Aggelis *et al.*, 2003). Laccase was found to be responsible for the transformation of 2,4,6-

trichlorophenol to 2,6-dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone (Menale *et al.*, 2012).

In the presence of mediator such as ABTS and HBT, laccase from white rot fungi have also been used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene (Niku-Paavola and Viikari, 2000). The study of the laccase-mediator system in the bioremediation of polycyclic aromatic hydrocarbon (PAH) has been extensively reported (Dai *et al.*, 2011; Mayolo-Deloisa *et al.*, 2011).

2.4.4 Laccase Based Biosensor

Laccase-based electrochemical biosensors have been developed which enable the amperometric determination of phenolic compounds in waste streams (Freire *et al.*, 2002; Torrecilla *et al.*, 2007) or the quantitative estimation of wide range of aromatic substances and reducing compounds (Zouari *et al.*, 1994). In food industry, laccase-based biosensor has been used to detect phenol or other enzymes in fruit juice and wine (Di Fusco *et al.*, 2010; Gil and Rebelo, 2010).

Oxygen determination using laccase have been investigated (Gardiol *et al.*, 1996). Electrochemical studies using laccase cathodes allow the electro-reduction of oxygen to water (Pita *et al.*, 2011) which eventually was used in bio-fuel cells (Jenkins *et al.*, 2012).

2.4.5 Synthesis of New Chemicals

Laccase is used for bioconversion of aromatic compounds and several other chemicals to oxidized products. The formation of radicals catalyzed by laccase can result in a reaction of parent molecules themselves (self-coupling) or with a second type of molecule (cross-coupling). Laccase have been used in the

polymerization of bisphenol A (Ji *et al.*, 2009), 1-naphthol (Ceylan *et al.*, 2008) and vinyl monomers (Tsujimoto *et al.*, 2001). Other laccase homomolecular coupling reactions were used for the synthesis of new imidazole compounds (Schäfer *et al.*, 2001). Laccase heteromolecular coupling reactions can be used as a method for modifying bioactive compounds or chemical syntheses (Mikolasch *et al.*, 2002).

2.4.6 Food Industry

Laccase can be applied to process that enhance or modify the colour appearance of food or beverage. It is assumed that phenolic saccharides and other phenolic compounds are responsible for browning. Laccase is set to be employed in the processing of juices to make clear and stable products (Neifar *et al.*, 2011; Stanescu *et al.*, 2012). In baking industry, laccase have been widely used due to its ability to cross-link biopolymers. Laccase from white rot fungus *Trametes hirsuta* increased the maximum resistance of dough extensibility in flour and gluten dough (Selinheimo *et al.*, 2006).

2.4.7 Others Laccase Application

Catalytic ability of laccase can be used to degrade contaminants in soil such as 2,4,6-trinitrotoluene (TNT) to an organic soil matrix which resulted in detoxification of the munition residue (Nyanhongo *et al.*, 2006). In the oil mining sites, some of the natural oil deposits are belong to the polycyclic aromatic hydrocarbon (PAH) group. The PAH arise from the oil deposits sites were found to be degraded by laccase (Pointing, 2001). Laccase-based hair dyes have been introduced which are less irritant and easier to handle compared to chemical hair dyes (Jeon *et al.*, 2010).

2.5 Production of Laccase by *Pycnoporus sanguineus*

White rot fungus *Pycnoporus sanguineus* have been studied extensively because they are one of the few organisms that can produce laccase (Pointing *et al.*, 2000; Garcia *et al.*, 2006). Their ability to degrade lignin into carbon dioxide and water are due to the laccase enzyme which is secreted by them. Laccase production by *Pycnoporus sanguineus* has been studied in many biotechnological potential applications such as decolorization of azo and anthraquinonic dyes (Lu *et al.*, 2007), Kraft pulp biobleaching (Eugenio *et al.*, 2010), bioconversion of rutin for cosmetic and pharmaceutical applications (Uzan *et al.*, 2011), textile wastewater treatment (Liu *et al.*, 2012), immobilization as nanoparticle composite (Huang *et al.*, 2006), etc. Fermentation technology has been applied extensively in the enzyme production and its optimization process. Laccase fermentation studies from white rot fungi have been performed in submerged fermentation, SMF (Garcia *et al.*, 2006; Songulashvili *et al.*, 2007; Tlecuitl-Beristain *et al.*, 2008; Flores *et al.*, 2010) and solid state fermentation, SSF (Sun *et al.*, 2009; Annuar *et al.*, 2010; Philippoussis *et al.*, 2011).

Solid state fermentation (SSF) is fermentation where molds and several mycelia fungi are cultivated on solid substrate at low moisture level. In submerged fermentation (SMF), organism is grown in liquid medium which contain dissolved oxygen and nutrients. Compared to solid state fermentation, moisture level of submerged fermentation is more than 95% (Shuler and Kargi, 1992). Due to limited water level, Hölker *et al.* (2004) concluded SSF is difficult to scale up because of the build-up of gradients in temperature, pH, moisture, oxygen, substrate and inoculum. The SSF processes are slower than submerged process due to the solid substrate that acts as a barrier and it tends to have heat transfer problems from the inter and intra particle resistance (Raghavarao *et al.*, 2003)

which makes it harder to control. According to Harvey and McNeil (1994) at the highest biomass level, the fermentation broth display shear thinning or pseudoplasticity. Cultures with filamentous fungal are usually exhibited high apparent viscosity and non Newtonian rheology (Casas López *et al.*, 2005; Wucherpfennig *et al.*, 2010). Due to the uncontrolled growth of fungal, good mass transfer is difficult to achieve. At the same time, the extensive growth of fungus in bioreactor can limit cultivation time due to operational problem. For certain enzymes, production rate was found higher at the end of fungal growth. Table 2.2 showed previous work on laccase production in STR at different fermentation conditions.

2.6 Culture Condition in Laccase Submerged Fermentation

Performance for laccase production system at large scale primarily depends on how good is the mixing and mass transfer within the process. Mixing and mass transfer are influenced by agitation and aeration. Mixing is required in order to obtain homogeneity in liquid medium. In large fermenter, two or more impellers are used. Several studies indicated that laccase production can be repressed by mechanical stress on the fungi. Laccase production was decreased when *Trametes multicolour* cultivated in STR suffered damage to the mycelia caused by shear stress (Hess *et al.*, 2002). The design of the STR should consider the level of aeration rate and agitator in order to minimize shear forces which cause damage to mycelia and diminish laccase expression (Majeau *et al.*, 2010).

Comparison studies on laccase production using three type bioreactor configurations; a STR, an air-lift reactor (ALR) (Liu *et al.*, 2013) and a rotary drum reactor (RDR) (Domínguez *et al.*, 2001) have been carried by Fenice *et al.* (2003). They found laccase activity was strongly affected by impeller speed,

resulting in low enzyme activity in STR as compared to ALR and RDR. Fenice *et al.* (2003) had reported that the lowest laccase activity was recorded at highest agitation rate. However, Tavares *et al.* (2006) had reported no significant changes in laccase production level.

Table 2.2: Laccase production by white rot fungi at reactor scale under different fermentation conditions

Fungus	**Volume (L)**	**Aeration**	**Agitation (rpm)**	**pH**	**Max. Activity (UL^{-1})**
Trametes pubescens (Galhaup *et al.*, 2002b)	15	0.1 - 1.25 vvm	100	Uncontrolled with initial pH 5.0	335,000
Phanerochaete flavido-alba (Blánquez *et al.*, 2002)	0.975	-	70	-	72
Pleurotus ostreatus (Aggelis *et al.*, 2003)	3	1.0 vvm	200	Uncontrolled with initial pH 6.0 ± 0.3	65
Pycnoporous cinnabarinus (Sigoillot *et al.*, 2004)	12	-	150	Controlled at pH 5.0	2800
Phanerochaete chrysosporium (Gnanamani *et al.*, 2006)	0.8	-	150	Controlled at pH 5.5	30.2
Panus tigrinus (D'Annibale *et al.*, 2006)	3	1.0 vvm	250	-	4,600 ± 98
T. versicolor (Tavares *et al.*, 2006)	1	-	100 - 180	Controlled at pH 5.2	11,403
T. versicolor (Thiruchelvam and Ramsay, 2007)	1.25	0.2 - 1 vvm	200 - 350	Controlled at 4.5 to 5.5	1,385
Panus tigrinus (Quaratino *et al.*, 2008)	2	1.0 vvm	500	-	2,200

Cultivation of fungus in submerged fermentation is associated with the production of pellets and mycelium. The different morphological growth forms of the filamentous have significant effect on the rheology of the fermentation broth which influences the performance of the bioreactor. Agitation has been reported to affect pellet size in *Pleurotus ostreatus* with pellets diameter reduced by 60% when agitation rate was set at 400 rpm (Márquez-rocha *et al.*, 1999). Pellets may be classified as stable spherical or oval agglomerates, consisting of branched and intertwined networks of hyphae (Braun and Vecht-Lifshitz, 1991). The growth of pellets which exceed a critical radius will cause difficulties for substrates to penetrate the dense core of the pellets. Thus, a typical pellet consists of three regions which are growing region at the outer shell, the non-growing region mycelia biomass and the hollow centre (Cui *et al.*, 1997; Cui *et al.*, 1998). The effect of agitation on fungal pellets morphology is described in Figure 2.4.

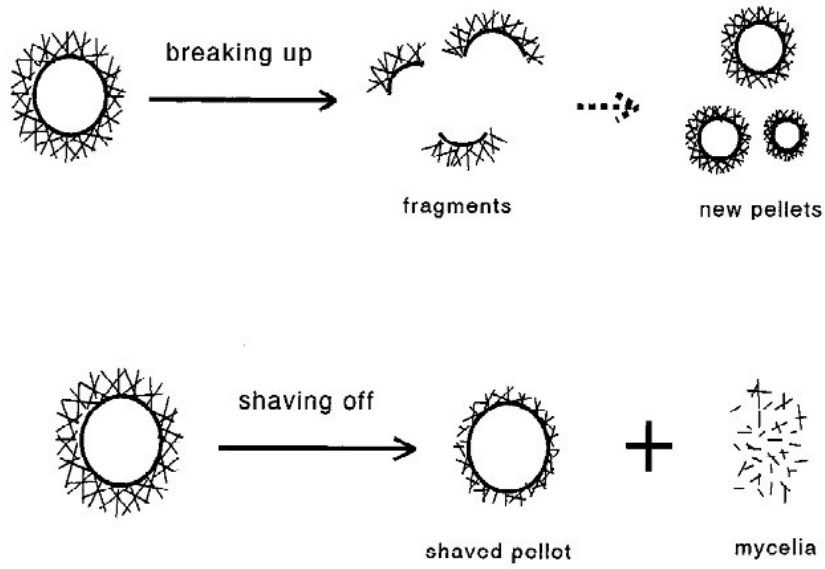


Figure 2.4: Schematic description of agitation effect on pellet (Adapted from Cui *et al*. (1997))

Aeration in STR is one of the parameters important for supplying oxygen through air bubbles. Several steps take place during oxygen transfer from air bubble to fungal cell. The oxygen must first travel through the gas–liquid interface, then the bulk of liquid and finally into the cell (Doran, 1995). The major driving force in the transfer of oxygen from gas and liquid interface to bulk liquid is concentration gradient of oxygen in the liquid medium. Despite the needs of oxygen in fungal growth and metabolism, oxygen also is required to remove carbon dioxide from the liquid due to fungal respiration.

The effect of pH medium also play vital role in laccase production by white rot fungi. Without pH regulation, cultivation of fungal culture will shift the pH medium to become more acidic or alkaline conditions. The effect of controlled pH in laccase productivity depend on differences in enzyme secretion time, stability of enzyme at different pH medium, protease production during the whole fermentation and sensitivity of a fungal response to abiotic stress (Janusz *et al.*, 2007).

Longer fermentation period is one of the main problems in the cultivation of fungus in STR due to bioreactor operational failures over extended period. In order to avoid this problem, fungal is immobilised to control biomass growth. The immobilised fungal cells are easy to separate from liquid medium. Furthermore, immobilised cells are protected from shear stress (Kong *et al.*, 2003).

2.7 Fermentation Medium for Laccase Production

Laccase enzyme production is limited by the glucose and nitrogen source in the fermentation. Laccase production occurs during secondary metabolism and is subject to complex regulation by nutrients such as carbon, nitrogen, inducers and copper during fungal growth which affects the transcription levels of laccase

genes in various fungal taxa (Pointing *et al.*, 2005). The correlation between nitrogen concentration in *T. versicolor* incubation and the level of *lcc* gene expression was observed by Collins and Dobson (1997). They found at high concentration of ammonium tartarate, laccase activity was high due to the increasing expression of *lcc* mRNA.

Nyanhongo *et al.* (2002) observed that *Pycnoporus cinnabarinus* was less sensitive to high concentration of nitrogen. The ratio of carbon to nitrogen (C/N) in the fermentation medium is one of the main effects in the laccase production. Suitable C/N ratio is important in order to obtain high laccase enzyme. If C/N ratio is very high, enzyme extraction from the medium is difficult due to the interference of other component such as extracellular polysaccharides. Incubation of *P. chrysosporium* in medium with high C/N ratio reduced the laccase activity due to the formation of *β*-1,3-glucan (Gutierrez *et al.*, 1996; Dekker and Barbosa, 2001). Biomass from *Botryosphaeria rhodina* did not statistically influenced by C/N ratio in the liquid medium when inorganic N was used as nitrogen source (Dekker *et al.*, 2007). When organic N (asparagine) at C/N ratio = 6.58, the levels of laccase produced were significantly higher than those using inorganic source (NH_4NO_3) when induced with vanillic acid.

The nature of N source for fungal cultivation has significant influents on production rate. In previous studies, the effect of organic and inorganic N sources has shown significant difference in laccase productivity. The effect of inorganic N sources (NH_4NO_3, $(NH_4)_2SO_4$ and $NH_4H_2PO_4$) and organic N sources (urea and proline) have increased laccase titers of *B. rhodina* culture in production medium with vanillic acid inducer (Dekker *et al.*, 2007). However, there was no significant effect of inorganic N source on laccase activity in medium without vanillic acid inducer (Dekker *et al.*, 2007).

Laccase formation was strongly affected by the nature of carbon source. Generally, carbon source that were efficiently and rapidly utilized by organism resulted in high levels of laccase activity. Simple type of carbon source such as glucose can be used as the carbon source for laccase production. In the large scale production, the application of natural carbon sources particularly contain lignocellulosic materials is significant as it might reduce the production cost. Table 2.3 indicates the application of several carbon sources for laccase production in submerged fermentation.

Table 2.3: Fungal laccase production using different carbon source

Species	**Carbon source ($g\ L^{-1}$)**	**Cultivation (days)**	**Lac. Activity ($U\ L^{-1}$)**
Botryosphaeria MAMB-5 (Dekker *et al.*, 2001)	10.0 Pectin	4.5	1440 ± 74
Botryosphaeria sp. (Cunha *et al.*, 2003)	10.0 Fructose	4.5	870 ± 20
Trametes versicolor (Tavares *et al.*, 2006)	11.0 Glucose	9	11403
Pestalotiopsis sp. (Hao *et al.*, 2007)	10.0 Maltose	12	1800 ± 200
Trametes pubescens CBS 696.94 (Osma *et al.*, 2007)	10.0 Glycerol	16	89
Pleurotus sajor-caju PS-2001 (Bettin *et al.*, 2009)	5.0 Sucrose	7	3170

The addition of inducers in the laccase production medium can stimulate the productivity of laccase in STR. Inducers for laccase production can be classified into aromatic compounds, metal ions and natural inducers. However,

some of the inducers are toxic to microorganism. Metal ions such as Ag^{+}, Cd^{+} and Hg^{+} are highly toxic for the microorganism due to complete inhibition of growth after addition (Galhaup *et al.*, 2002a). Furthermore, the effect of metal ions such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Ca^{2+} promoted laccase production by *Polyporus* sp. while the addition of Co^{2+}, Al^{3+}, Cu^{2+} and Fe^{2+} inhibited enzyme activity (Guo *et al.*, 2011). The used of aromatic inducers such as gallic acid, catechol, *p*-anisidine, guaicol, ferulic acid, 2,5-xylidine and vanillic acid were significantly increased laccase activity (Galhaup *et al.*, 2002b; Pazarlioğlu *et al.*, 2005).

2.8 Basic Concept of Design of Experiment (DOE)

Statistical design of experiment refers to the process of planning the experiment so that appropriate data can be analyzed by statistical methods resulting in valid and objective conclusions (Montgomery, 2001). When dealing with experimental data, there are problems due to experimental errors. The only approach to overcome problem associated with experimental error is through statistical methodology.

It is important for researchers to have clear idea before proceed with actual experiments. Figure 2.5 showed the guidelines for designing an experiment suggested by Montgomery (2001). Even if the data is collected in the right way, the good analysis will depend on how the whole experiment is done. Without proper experimental design, it is difficult to archive clear and precised analysis.

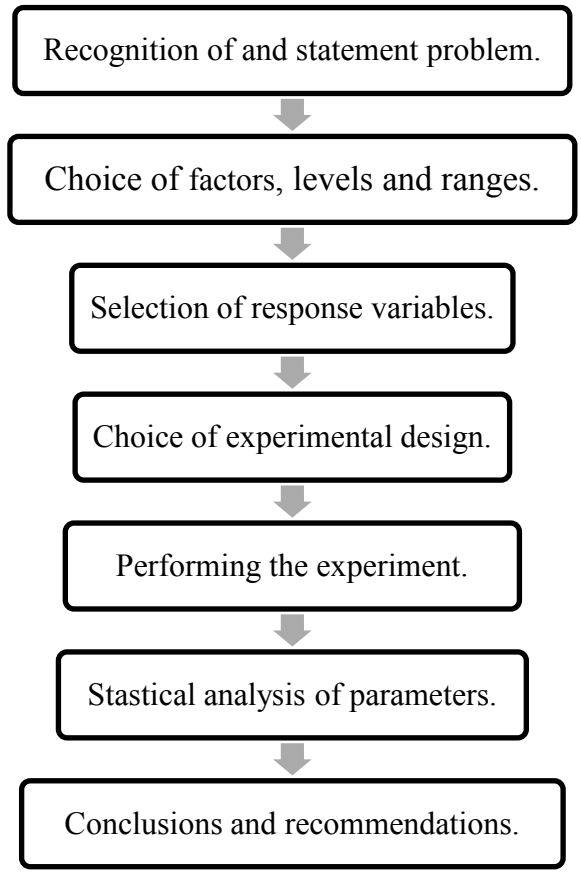


Figure 2.5: Experimental design guidelines (Adapted from Montgomery (2001))

When designing an experiment, the whole experiment must be clearly understood. Level for each factor of interest must be considered based on practical experience and theoretical understanding. The effect of nuisance factor such as control, uncontrollable and noise factor must be classified before proceeding with actual experimental runs. Each of the experimental design has different set of goals which is depending on the research objective(s) (Table 2.4).

Table 2.4: Design selection based on experiment objective and factors of interest

Experimental design	**Objective**	**No. of factor(s)**
One factor completely randomized design	Comparatives	1
Randomized block design	Comparatives	≥ 2
Fractional factorial design	Screening	2 – 4
Full factorial design	Screening	2 – 4
Plackett-Burman design	Screening	≥ 5
Central composite design	Optimization	2 – 4
Box Behnken design	Optimization	2 – 4

2.9 Full Factorial Design

Factorial design is one of the common statistical methods that used to analyze two or more factors. The effect of a factor in factorial design is defined as the response changes produced by a change in the level of the factor (Montgomery, 2001). There are two types of effect in the factorial design; (i) main and (ii) interactions effect. Main effect is referring to primary factor of interest in the experiment. An interaction effect is the variation of differences between means for different levels of one factor over different levels of the other factor. The difference between main and interaction effect are described (Figure 2.6 A and B).

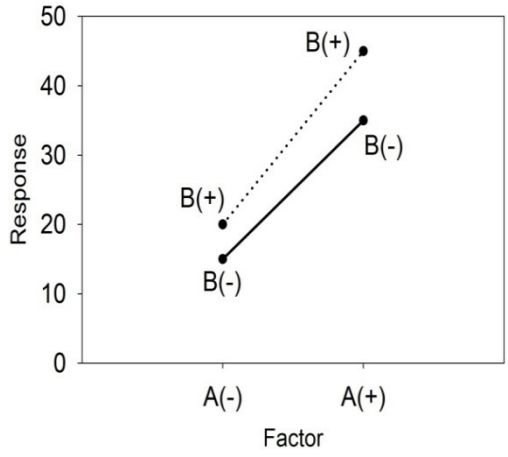


Figure 2.6(A): A factorial experiment without interaction

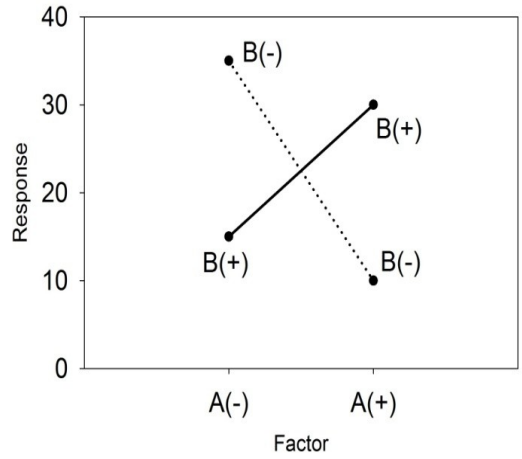


Figure 2.6(B): A Factorial experiment with interaction

In factorial design, all input factors are set at two levels each; high (+1) and low (-1). Factorial design can be divided into full factorial (2^K) and fractional factorial (2^{K-1}). In this design, the total number of experiments for studying k factors at two levels (2-level) is 2^K. A design with complete replicates of all possible combinations of input factors is called a full factorial design. Fractional factorial design is a design which made up from a fraction of the complete factorial experiment (Montgomery, 2001). Although fractional factorial design

requires only few runs compare with full factorial, some consideration must be considered since there are confounded factors in the fractional design. The 2-level design is often used to provide direction for further experimentation. For example, if there is possible region where the optimal settings may exist, factorial design can be extended to form a central composite design.

2.10 Central Composite Design (CCD)

Central composite design (CCD) is a common response surface experiment consists of a combination either factorial or fractional design with centre points and axial points, allows the estimation of curvature in response surface design. A CCD is characterized by the orthogonal blocking allowing for model terms and block effects to be randomly estimated with smaller variation in the regression analysis. In addition, the CCD provides a constant prediction variance at all points that are equidistant from the design centre which refer to the rotatability of a design. Generally the CCD consists of:

1. 2^K factorial (or fractional factorial of resolution V),
2. $2K$ axial or star runs,
3. Centre points.

where K is number of factors. Figure 2.7 shows the design points for the CCD experiment with $K = 2$ factors.

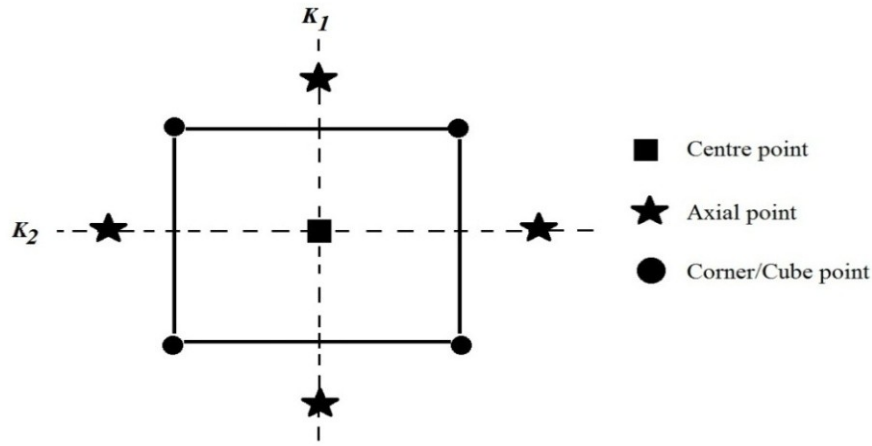


Figure 2.7: CCD design points for no. of factor K = 2

Rotatability is a property with unchanged variance when the design is rotated about the centre which determined by the value of α with $\alpha = (n_F)^{1/4}$, n_F is the number of corner/cube points of the factorial portions (Montgomery, 2001). A spherical CCD is a rotatable design obtained when $\alpha = (K)^{1/2}$, K is the number of factor (Montgomery, 2001). The choice of α depends on the factor region of interest. In some cases, the factor setting is out of range which is impossible to perform in real experiment. For example, at default alpha value (alpha = 1.414) the agitation setting for corner/cube points of 100 and 600 rpm resulted in axial points of -3.6 and 703.5 rpm, respectively. The choice factor setting has to be considered in order to obtain rotatable design with default α value.

2.11 Face-Centred Central Composite Design (FCCCD)

When $\alpha = 1$, the region of interest is not in the form of spherical. In this case, the CCD with $\alpha = 1$ is known as the face-centred central composite design (FCCCD). In this design, the axial points are located at the centre of each face of factorial space (Figure 2.7). This design required only three levels of each factor. By augmenting the existing points in factorial (or fractional with resolution V)

design with the axial points can also produce the FCCCD. However, the main drawback of FCCCD is the design is not rotatable and difficult to change the factor level without introduce large variance when rotating about the centre. In simple term, the FCCCD is suitable for the process limited to the factor region of interest.

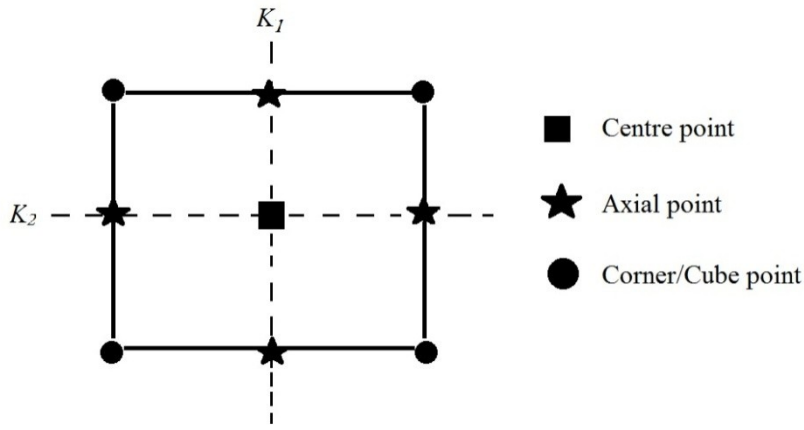


Figure 2.8: FCCCD design points for no. of factor K = 2

2.12 Screening and Optimizing Process Parameter for Laccase Production

In some process development, the number of potential factors (design parameters) is large. Screening is used to reduce the number of factors by identifying only the significant factors which affect product quality. Various design such as factorial design, Plackett Burman Design, fractional factorial design, etc., can be used for preliminary studies. The 2^K factorial design is particularly useful in the early stages of process development. For example if there are seven factors of interest, the factorial design is used to screen out of which factors are significant. Meanwhile, the Plackett Burman design offers a design with n variables are studied in $n + 1$ experimental runs with only require fewer runs than a comparable fractional design. Hence the numbers of runs for PBD are available in multiples of four runs which are excellent for screening purpose. The orthogonal design of Plackett Burman assumes that two factors interactions are

negligible and pure effect of each variable not confounded with the interaction effects (Montgomery, 2001; Chauhan *et al.*, 2007).

However, the used of traditional one factor of time method was used initially for determination of optimum concentrations before furthered optimized using response surface method (Nyanhongo *et al.*, 2002). This conventional method is time consuming and unable to estimate interactions among factors results in over estimating of the optimal point. The preliminary study experiments using full factorial design (FFD) have been reported for laccase optimization studies (Vasconcelos *et al.*, 2000; Liu *et al.*, 2009). Furthermore, the application Plackett Burman Design (PBD) accompanied by response surface optimization of laccase production by fungal have been studied (Arockiasamy *et al.*, 2008). As previously mentioned, The significant factors obtained from the preliminary studies was furthered employ in the optimization process by response surface method using central composite or Box-Behnken (Montgomery, 2001).

2.13 Bioprocess Modelling of Laccase by White Rot Fungi

Mathematical modelling is a very powerful tool in many fermentation processes by microorganism for interpretation and prediction of natural phenomena and experimental results (Gershenfeld, 1999). In nature, a fungal cell activity consists thousands enzymes catalysing different biochemical reactions, growth and production formation resulted in complex interactions (Brass *et al.*, 1997). Modelling process in fungal fermentation can be classified in two large groups consist of mechanistic and empirical models (Gernaey *et al.*, 2010).

An empirical model represents input-output relations in a data set without requiring detailed knowledge about a process. A mechanistic model is a mathematical formulation of the internal process in terms of its constituent parts

and mechanisms (Nielsen *et al.*, 2003). For example in laccase fermentation, the main constituents are substrate, oxygen, reactor volume, inducers, etc., whereas the main mechanisms are microbial growth, mass transfer (aeration and agitation) and mixing (substrate, pH control).

Understanding of kinetic modelling in fermentation process is crucial for bioprocess development. The combination of experimental data with mathematical modelling provides meaningful interpretation in terms of quantitative information as well as qualitative observation. The kinetic modelling in fungal fermentation is difficult and challenges exist at all stages of process development including model formulation, identification and parameters estimation, and solution of the equations (Coppella and Dhurjati, 1989; Thilakavathi *et al.*, 2007).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Microorganism and Culture Maintenance

A white rot fungus, *Pycnoporus sanguineus* was obtained from University of Malaya Fungal Culture Collection. The fungal was cultured in potato dextrose agar (PDA) plates containing 1% (w/v) of coconut husk for seven days before being transferred to GYMP (glucose-yeast-malt-peptone) agar plates. The coconut husk was used to induce the production of laccase enzyme (Karim and Annuar, 2009). Table 3.1 showed the components of GYMP agar medium.

Table 3.1: Glucose-Yeast-Malt-Peptone (GYMP) agar medium

Components	**g L^{-1}**
Glucose	20.0
Yeast Extract	2.0
Malt Extract	2.0
Peptone	2.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	0.46
K_2HPO_4	1.0
Agar Technical No. 3	19.0

Fungal culture in agar plates was incubated at 28 ± 2 °C for 7 days (Plate 3.1). For long term storage, the fungus was stored in 10 % (v/v) glycerol at 4 °C and sub-cultured every three months. Figure 3.1 summarized the steps in the preparation *P. sanguineus* culture in agar plates.

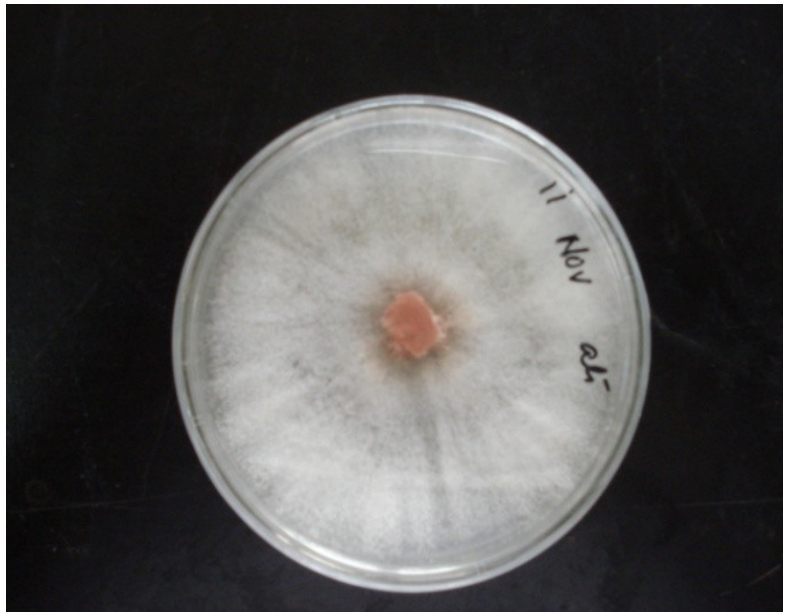


Plate 3.1: Seven-day old culture of *P. sanguineus* in GYMP agar medium

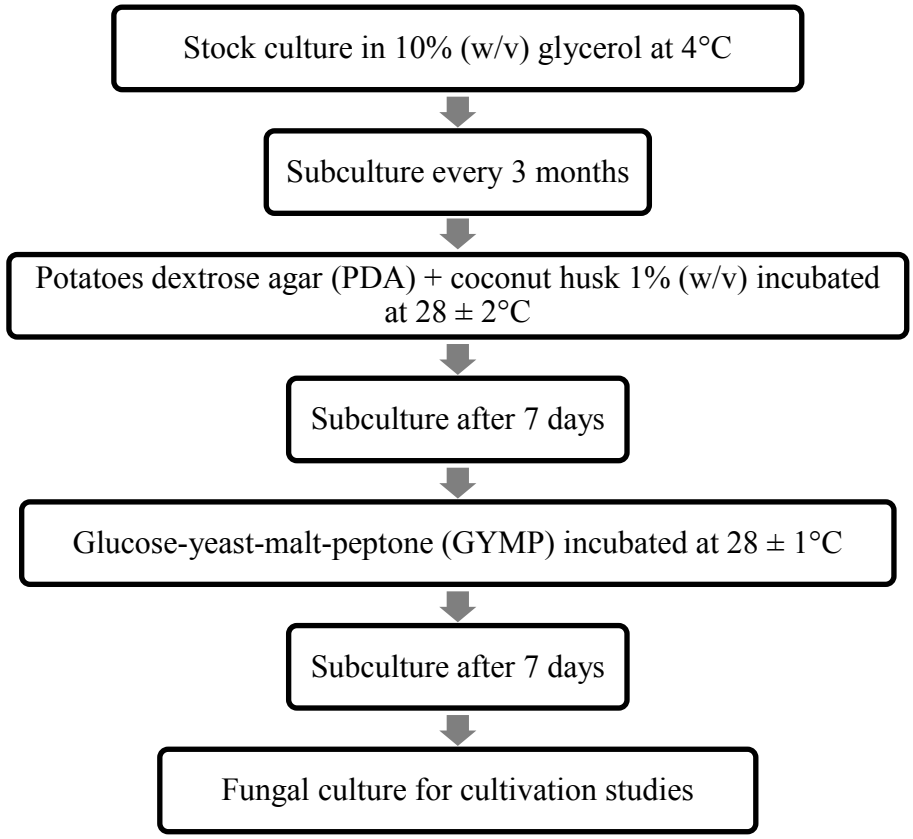


Figure 3.1: Preparation of *P. sanguineus* plate culture

3.2 Inoculum Preparation for Agar Plate and Shake Flask Cultivation

For the agar plate cultivation, a mycelia disc (9.0 mm in diameter) was harvested from the growing edge of the mycelium (Plate 3.1). In the case of shake flask cultivation, three mycelium discs (9.0 mm in diameter) from 7 days plate culture were used as inoculum in the flask cultivation.

3.3 Improvement of Liquid Medium for Submerged Fermentation (SMF)

Screening experiment was done to determine the best liquid medium for maximum enzyme productivity. It was performed using original basal medium (Table 3.2) and by adding different types of complex nutrient and glucose concentration. Three concentrations of glucose anhydrous (5, 10 and 25 g L^{-1}) were tested. For complex nutrient, yeast extract (Becton-Dickinson), malt extract (Becton-Dickinson) and peptone (Becton-Dickinson) were added at the concentration of 2.0 g L^{-1} each.

Table 3.2: Original basal liquid medium

Components		**g L^{-1}**
Carbon source	Glucose	20.0
Complex nutrient	Malt extract	2.0
Salt	$(NH_4)_2SO_4$	2.0
	KH_2PO_4	0.5
	$MgSO_4.7H_2O$	0.1
	CaCl	0.05
	KCl	4.0
	$CuSO_4.5H_2O$	0.01

Experiments were performed in two stages. The first stage was factorial experiments on different complex nutrients added. The effect of yeast extract, malt extract and peptone on laccase production was evaluated using 2-level full factorial design (FFD). The amount of other components was fixed according to the original basal medium. Statistical software Minitab 16 was used to design the

FFD experiment. All factors and levels set up were summarized in Table 3.3. Extracellular enzyme production after seven days was used as the response in the experiment. Fermentation was performed in 250 mL Erlenmeyer flasks containing 100 mL of tested medium. The flasks were shaken using orbital shaker (Lab Tech, Korea) at agitation speed of 170 rpm, 25 ± 2 °C. Three fungal mycelium discs (9.0 mm diameter) from the growing peripheral of fungal agar plate culture were used as inocula.

Table 3.3: Factor and level set up for improvement of liquid medium experiments in shake-flasks

Factor	**Level**	
	Minimum (-1)	**Maximum (+1)**
Yeast extract (g L^{-1})	0.0	2.0
Malt extract (g L^{-1})	0.0	2.0
Peptone (g L^{-1})	0.0	2.0

The second stage experiment investigated the effect of initial glucose concentration on laccase production. Three glucose concentrations *viz* 5.0, 10.0 and 25.0 g L^{-1} were tested based on the result obtained from the first stage FFD experiment. Fermentation experiments were carried out under similar conditions as in the first stage. Formulated medium obtained from these experiments was used for the production of enzyme in the shake flasks and stirred tank reactor (STR).

3.4 Laccase Production in Shake Flasks

Enzyme production in shake flasks was conducted in 250 mL Erlenmeyer flask containing 100 mL medium. Shake flask cultivation studies will include the effect of baffled and non baffled flasks on laccase production at agitation speeds of 100, 170 and 250 rpm. The schematic diagram and dimension of the baffled flask

used in the study is shown in Appendix A. Cultivation was carried out using Lab Tech (Korea) orbital shaker at 25 ± 2 °C. The culture medium was inoculated with three fungal mycelium discs (9.0 mm diameter) from the 7 days old GYMP agar culture. Laccase activity, biomass dry weight, glucose concentration and protein content were determined in order to obtain the fermentation profile of laccase production in the shake flasks.

3.4.1 Theoretical Estimation of Oxygen Mass Transfer Coefficient (k_La) in Shake Flask

According to Henzler and Schedel (1991), the k_La value can be increased with higher shaking frequency. In the current study, the increased of shaking frequency would influence the production of biomass and laccase production. The estimation of k_La in shake flasks was determined theoretically using equation described by Henzler and Schedel (1991):

$$k_L a = \frac{0.5 d^{73/36} \times n \times d_0^{1/4} \times D^{1/2}}{V_L^{8/9} \times v^{13/54} \times g^{7/54}} \qquad \text{Equation 3.1}$$

where k_La is mass transfer coefficient (s^{-1}), d is maximum inner flask diameter (m), n is shaking frequency (s^{-1}), d_0 is shaking diameter (m), V_L is filling volume (m^3), D is diffusion coefficient of oxygen in water at 25 °C ($m^2\ s^{-1}$), v is kinematic viscosity of water at 25 °C ($m^2\ s^{-1}$) and g is gravitational acceleration ($m\ s^{-2}$).

3.5 Laccase Production in Stirred Tank Reactor (STR)

Laccase production in stirred tank reactor (STR) was carried out in a double jacketed 2-liter working volume Sartorius Biostat-B Plus (Germany) fermenter using laccase production medium. The composition of laccase production medium

is obtained from screening experiment in Section 3.3. The total volume of fermentation medium was 1.2 L and pH was adjusted before autoclaving using potassium hydroxide (KOH) and hydrochloric acid (HCl). Bioreactor was sterilized using an autoclave (TOMY SS-325, Japan) at 121 °C for 15 minutes. Glucose was autoclaved separately. In order to induce laccase production, copper sulphate pentahydrate ($CuSO_4.5H_2O$) was added into the system. Copper sulphate stock solution of 10 g L^{-1} was prepared by filter sterilizing method. A total volume of 1.2 ml inducer was added from stock solution into the fermentation medium at final concentration of 0.01 g L^{-1}. Both glucose and inducer were aseptically added into the sterile bioreactor prior to the start of the fermentation.

The fermenter set up consists of probes for (i) temperature (Pt-100, Germany), (ii) pH (Mettler Toledo Model: 405-DPAS-SC-K8S/200, Switzerland) and (iii) partial oxygen (Mettler Toledo Model InPro, Switzerland). The fermenter was equipped with a Rushton turbine impeller, four baffles and a sparger ring. To avoid foam formation, a modified mechanical foam breaker was used. Schematic diagram and the geometric characteristic of the fermenter is shown in Appendix B.

3.5.1 Estimation of Oxygen Mass Transfer Coefficient (k_La) in Stirred Tank Reactor (STR)

The k_La value for STR cultivation was determined by using static gassing out method which involves monitoring and measuring dissolved oxygen increment during oxygenation and mixing operations. Oxygen was monitored in the form of oxygen partial pressure using polarographic oxygen probe.

The methodologies for the static gassing out method involve the purging of dissolved oxygen from fermentation liquid by continuously sparging nitrogen gas until probe reading shows zero (0 % pO_2). After zero reading stabilizes, nitrogen

gas was shut off and air was added immediately until pO_2 started to increase and stabilize. At this stage, the increment of % pO_2 reading (C_L) was recorded for every 5 seconds until it stabilized. The fermentation medium as indicated in Section 3.3 was used for the k_La determination in STR. Under steady state condition, the rate of oxygen mass transfer was given by:

$$\frac{dC_L}{dt} = k_L a\left(C_E - C_L\right) \qquad \text{Equation 3.2}$$

where dC_L/dt is the rate of oxygen transfer (s^{-1}), C_L is the dissolved oxygen concentrations, C_E is the dissolved oxygen concentration in equilibrium, t is the time (s) and k_La is oxygen transfer coefficient (s^{-1}). Integration of Equation 3.2 yields:

$$\ln\left(C_E - C_L\right) = -k_L a \times t \qquad \text{Equation 3.3}$$

which the plotted graph of ln (C_E - C_L) as function of t will results a linear plot with the slope equals to k_La value (-m = -k_La).

3.6 Inoculum Development for Stirred Tank Reactor (STR) Fermentation

Inoculum for STR fermentation was cultivated in 250 mL Erlenmeyer flask containing 100 mL of GYMP growth medium (Low *et al.*, 2008). The components of GYMP growth medium is shown in Table 3.4. Three mycelia discs (9.0 mm in diameter) from the growing edge were used as the inocula. Inoculum cultivation was carried out using orbital shaker (Lab Tech, Korea) at agitation speed 170 rpm, 25 ± 2 °C for 4 days.

After 4 days, the total content of inoculum culture (100 mL) was filtered using sterile filter paper Whatman No. 42 under low vacuum pressure. Culture filtrate was removed and biomass retentate was washed with 200 mL sterile distilled water. Distilled water was filtered out and biomass retentate was re-suspended in 100 mL of sterile culture medium used in the STR cultivation. The 100 mL biomass suspension was then added into the sterile fermenter.

Table 3.4: Glucose-Yeast-Malt-Peptone (GYMP) growth medium components

Components	**g L^{-1}**
Glucose	20.0
Yeast Extract	2.0
Malt Extract	2.0
Peptone	2.0
$MgSO_4.7H_2O$	0.5
KH_2PO_4	0.46
K_2HPO_4	1.0

3.7 Screening of Selected Operating Variables for Laccase Production in Stirred Tank Reactor (STR)

The effects of three selected operating variables namely agitation rate (rpm), aeration rate (L min^{-1}) and pH were screened using 2-level full factorial design (FFD). Laccase production in STR was performed in random triplicates with a minimum and maximum factor level (Table 3.5). Three centre point experiments were added into the FFD analysis in order to detect curvature (Montgomery, 2001). The fermenter was operated on automatic addition of 2.0 N KOH and 0.1 N HCl in order to control the pH at desired value. Laccase production after 5 days was used as the response for FFD analysis. All data was analyzed using ANOVA with first order polynomial used to fit the experimental data.

Table 3.5: Factor and level set up for FFD screening experiment in STR

Factor	Level	
	Minimum (-1)	**Maximum (+1)**
Aeration rate (L min-1)	1.0	1.5
Agitation speed (rpm)	100	600
pH	3.0	5.0

Fermentation was carried out at controlled temperature 28 °C for 5 days. Fermentation broth was harvested and filtered using Whatman No. 42 filter paper with the help of vacuum suction to remove cellular debris. The supernatant of each sample was assayed for laccase enzyme activity, protein and residual glucose. Fungal biomass estimation in STR cannot be performed directly by drawing sample from the fermenter's sampling port due to the nature of the fungal growth. Thus biomass amount in the STR cultivation was determined by harvesting the whole volume of the culture at the end of fermentation.

3.8 Optimization of laccase production in Stirred Tank Reactor (STR) using Face-Centred Central Composite Design (FCCCD)

Based on screening experiments in Section 3.7, the significant factors were further optimized *via* face-centred central composite design (FCCCD) where default α value is equal to 1. By adding axial and centre points to the existing cube points in the factorial design, a face-centred central composite design can be produced (Montgomery, 2001; Rudaz *et al.*, 2001). Statistical software Minitab 16 was used to design the experiments. With face-centred design, the experimental data can be modelled using the general form of quadratic equation shown:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{12} AB \qquad \text{(Equation 3.4)}$$

where Y is the response of experiment, A and B are factors, β_0 is coefficient constant, β_1 and β_2 are linear term coefficients for each factor, β_{11} and β_{22} are squared terms coefficients for each factor and β_{12} is coefficient constant for interaction between two factors.

3.9 Analytical Methods

All samples from fermentations in shake flasks or STR were filtered using Whatman filter paper No. 42 and PES (polyethersulfone) syringe filter 0.22 μm before performing any analytical assays.

3.9.1 Laccase Activity Assay

Laccase activity was determined by measuring the absorbance changes due to oxidation of syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) in sodium citrate buffer (pH 4.8) at 525 nm (ε = 65,000 M^{-1} cm^{-1}). The reaction mixture contained 0.2 mL filtered sample, 0.2 mL of 0.1 mM syringaldazine in 50 % (v/v) ethanol and 3.0 mL of 50 mM sodium citrate buffer pH 4.8. Reaction was carried out at controlled temperature of 25 °C.

Substrate 0.1 mM syringaldazine in 50 % (v/v) ethanol was prepared by diluting the stock solution, 1.0 mM syringaldazine in 95% (v/v) ethanol. Syringaldazine (Sigma) was dissolved in 95 % (v/v) ethanol and stirred for 2 to 3 hours using magnetic stirrer. To maintain substrate stability, 0.1 mM syringaldazine in 50 % (v/v) ethanol was prepared every month by diluting from the stock solution. All reagents were prepared using ultrapure water and stored at 4 °C. Sodium citrate buffer was prepared according to Henderson-Hasselbalch equation (Appendix C).

Enzyme activity assay was carried out by adding 0.2 mL of filtered sample into 3.0 mL of 50 mM sodium citrate buffer pH 4.8 and mixed gently. Then, 0.2 mL of 0.1 mM syringaldazine in 50 % (v/v) ethanol was added and mixed immediately at temperature 25 °C. All colorometric assays were done using JASCO V-630 (Japan) UV-VIS spectrophotometer. Enzyme activity (U L^{-1}) was determined using Jasco Spectra Manager software. Enzyme activity was calculated as follows:

$$\text{Enzyme activity } (\text{U L}^{-1}) = \frac{\Delta A}{\Delta t} \times \frac{1}{l} \times \frac{1}{\varepsilon} \times \frac{v_t}{v_s} \qquad \text{(Equation 3.5)}$$

where $\Delta A/\Delta t$ is absorbance changes per minute (min^{-1}), ε is syringaldazine molar extinction coefficient (65 000 M^{-1} cm^{-1}), v_t is total assay volume (L), v_s is total enzyme sample (L) and l is the light path length (1 cm). Thus 1 U enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol syringaldazine per minute at 25 °C. Specific enzyme activity was calculated using equation below:

$$\text{Specific activity } (\text{U g}^{-1}) = \frac{\text{Laccase activity (U L}^{-1})}{\text{Protein content (g L}^{-1})} \qquad \text{(Equation 3.6)}$$

3.9.2 Glucose Concentration

Residual glucose content in the sample from the fermentation process was determined using dinitrosalicylic acid or DNS method (Miller, 1959). In this method, the presence of free carbonyl group (C=O) referring to reducing sugars was tested. Aldehyde functional group in glucose was oxidised to form carboxyl group with 3,5-dinitrosalicylic acid reduced to 3-amino-5-nitrosalicylic (Hostettler *et al.*, 1951).

The composition and preparation of DNS reagent is shown in Appendix D. For a single assay, 3.0 mL DNS reagent was added into reaction mixture containing 3.0 mL of diluted sample. Then reaction mixture was vortexed before incubation in the waterbath at 95 °C for 15 minutes. After 15 minutes, 1 mL of Rochelle salt was added in order to stabilize the colour formed. The mixture was then cooled down using running tap water until room temperature. Absorbance at 575 nm was taken and glucose content was determined from the glucose standard calibration. All reagents were prepared using ultrapure water and kept cooled at 4 °C in the amber bottle.

3.9.2.1 Preparation of Glucose Standard Calibration

D-glucose (Systerm) at different concentrations (g L^{-1}); 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40 were dissolved in ultrapure water and used as standards. All measurements were done in triplicates and absorbance at 575 nm for each assay was plotted against glucose concentration. Glucose standard calibration is shown in Appendix D.

3.9.3 Protein Content

Lowry method was used to determine protein content in the fermentation samples. Two types of reagents i.e. Lowry reagent (Sigma) and Folin & Ciocalteu's phenol reagent (Sigma) were used in the assay. All reagents were prepared according to the supplier's instruction. The stock solutions were stored at room temperature in dark condition.

All samples were filtered using PES (polyethersulfone) syringe filter 0.22 μm with low protein binding characteristic. Protein content was determined by adding 1.0 mL of sample with 1.0 mL of Lowry reagent. Reaction mixture was

incubated at room temperature for 20 minutes. Then, 0.5 mL of Folin & Ciocalteu's phenol reagent was added immediately into the reaction mixture and incubated for 30 minutes. Absorbance at 750 nm was read and protein content was determined using protein standard.

3.9.3.1 Preparation of protein standard calibration

Bovine serum albumin or BSA (Sigma) was used as protein standards. BSA standard was prepared in different concentrations (in g L^{-1}); 0.02, 0.03, 0.04, 0.06, 0.08, 0.10, 0.15 and 0.20. All reagents and solutions were prepared using ultrapure water. BSA standard calibration plot is shown in Appendix E.

3.9.4 Biomass Estimation

Biomass was determined by gravimetric method. Since fungal growth is associated with heterogeneous growth, direct estimation by sampling small volume of culture medium was impossible. The only way to determine fungal biomass was to harvest the whole culture medium. Sample from the fermentation was filtered using pre-weighed Whatman No. 44 filter paper with the help of vacuum pressure. Filtrate was discarded and filter paper with biomass retentate was dried in the oven at 60 °C for 24 hours. Before weighing, the dried biomass on filter paper was taken out from the incubator and allowed to equilibrate to room temperature for 30 minutes in order to obtain constant reading. Analytical balance (Sartorius) with resolution of 0.1 mg was used to weigh biomass samples.

3.10 Kinetic Modelling of Laccase production

Kinetic model of batch fermentation for laccase production in shake flasks and stirred tank reactor was studied. Fermentation profile such as biomass (X),

laccase production (*P*) and glucose utilization (*S*) were used for the kinetic studies. The differential equation for each variable was integrated using Matlab 7 software. All kinetic parameters were calculated using non linear regression method with Levenberg-Marquardt (LM) algorithm (Polymath 6.0 software). Confidence intervals for the kinetic parameters were determined from the replicates of batch experiment. Based on the calculated parameters, unstructured kinetic model for laccase fermentation was developed. Simulation using Polymath 6.0 was performed by solving the differential equation of each kinetic model. The profile from the simulation was used to fit the experimental data.

3.10.1 Kinetic of Fungal Growth

The kinetic model for fungal growth was described according to Velhurst-Pearl logistic model (Equation 3.7):

$$\frac{dX}{dt} = \mu_{max}\left(1 - \frac{X}{X_{max}}\right)X \qquad \text{(Equation 3.7)}$$

where *dX/dt* is the rate of biomass production (g L^{-1} day^{-1}), *X* is the biomass concentration (g L^{-1}), μ_{max} is maximum specific growth rate (day^{-1}) and X_{max} is the maximum attainable biomass concentration (g L^{-1}). Maximum specific growth rate (μ_{max}) in the Equation 3.11 is depending on the fermentation condition such as temperature, pH, water activity and salt content (Zwietering *et al.*, 1990). The effects of limited nutrients concentration and unfavourable materials such as waste from cell metabolism can influence the X_{max} value. The experiment to determine X_{max} value was done by cultivating the cell until growth reaches plateau (*dX/dt* = 0).

3.10.1.1 Derivation of Logistic Growth Equation

The differential form of Equation 3.7 is rearranged as follows:

$$\frac{dX}{X}\left(\frac{1}{1-\frac{X}{X_{max}}}\right)=\mu_{max}\,(dt)$$

Introduce new variable, *K*:

$$K=\frac{X}{X_{max}}$$

$$X=K(X_{max})$$

$$\frac{dK(X_{max})}{K(X_{max})}\left(\frac{1}{1-\frac{K(X_{max})}{X_{max}}}\right)=\mu_{max}\,(dt)$$

Cancelling constant factor and rearrange the equation:

$$dK\left(\frac{1}{K(1-K)}\right)=\mu_{max}\,(dt) \qquad \text{(Equation 3.8)}$$

Partial Fraction Equation

The left side of Equation 3.8 can be expressed as partial fractions expansion where *A* and *B* are unknown constants:

$$\frac{1}{K(1-K)}=\frac{A}{K}+\frac{B}{(1-K)} \qquad \text{(Equation 3.9)}$$

Multiply with $K(1-K)$:

$$1=A(1-K)+B(K)$$

$$1 = A - A(K) + B(K)$$

$$1 = A + (B - A)K$$

$$1 + 0K = A + (B - A)K \qquad \text{(Equation 3.10)}$$

From the polynomial form in Equation 3.10 constants *A* and *B* can be determined as follows:

$$0 = (B - A)$$

where constant *A* can be determined directly from Equation 3.10:

$$A = 1$$

Therefore *B* is:

$$0 = (B - 1)$$

$$B = 1$$

Equation 3.8 is integrated using the partial fraction equation and substituted with constant *A* and *B*:

$$\int_{K_0}^{K} \left(\frac{1}{K} + \frac{1}{(1-K)}\right) dK = \mu_{max} \int_{0}^{t} (dt)$$

Since initial *t* is *t* = 0, therefore:

$$\ln|K|_{K_0}^{K} - \ln|1 - K|_{K_0}^{K} = \mu_{max}\,(t)_0^t$$

$$\ln\left(\frac{|K|}{|1-K|}\right)_{K_0}^{K} = \mu_{max}\,(t - 0)$$

$$\ln\left(\frac{|K|}{|1-K|}\right) - \ln\left(\frac{|K_0|}{|1-K_0|}\right) = \mu_{max}\,t$$

$$\ln\left(\frac{|K||1-K_0|}{|K_0||1-K|}\right) = \mu_{max}\,t \qquad \text{(Equation 3.11)}$$

Solve and rearrange Equation 3.11:

$$\frac{(K)(1-K_0)}{(K_0)(1-K)} = e^{\mu_{max}\, t}$$

$$\frac{(K)}{(1-K)} = \left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t}$$

$$K = (1-K)\left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t}$$

$$K = \left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t} - \left(\frac{K(K_0)}{1-K_0}\right) e^{\mu_{max}\, t}$$

$$K + \left(\frac{K(K_0)}{1-K_0}\right) e^{\mu_{max}\, t} = \left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t}$$

$$K\left(1 + \left(\frac{(K_0)}{1-K_0}\right) e^{\mu_{max}\, t}\right) = \left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t}$$

$$K = \frac{\left(\frac{K_0}{1-K_0}\right) e^{\mu_{max}\, t}}{\left(1 + \left(\frac{(K_0)}{1-K_0}\right) e^{\mu_{max}\, t}\right)}$$

$$K = \frac{K_0(e^{\mu_{max}\, t})}{(1-K_0) + K_0(e^{\mu_{max}\, t})} \qquad \text{(Equation 3.12)}$$

From Equation 3.12, there are two variables, K and K_0. Since $K = \frac{X}{X_{max}}$, K_0 is equal as:

$$K_0 = \frac{X_0}{X_{max}}$$

Therefore:

$$\frac{X}{X_{max}} = \frac{\frac{X_0}{X_{max}}(e^{\mu_{max}\, t})}{\left(1 - \frac{X_0}{X_{max}}\right) + \frac{X_0}{X_{max}}(e^{\mu_{max}\, t})}$$

Rearrange:

$$\frac{X}{X_{max}} = \left(\frac{1}{X_{max}}\right)\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})}$$

$$X = \frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})}$$

The final solution for the logistic equation is written as follow:

$$X = \frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})} \qquad \text{(Equation 3.13)}$$

3.10.2 Kinetics of Laccase Production

Kinetics of laccase formation is modelled using the Luedeking-Piret model which was previously used for the kinetics of lactic acid production (Luedeking and Piret, 1959). The equation contains coefficients describing the products partitioned into growth or non-growth associated phase. The general equation for Luedeking-Piret (LP) model is expressed as:

$$\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta X \qquad \text{(Equation 3.14)}$$

where *dP/dt* is the rate of laccase production (U L^{-1} day^{-1}), *dX/dt* is the rate of biomass production (g L^{-1} day^{-1}), α is the coefficients for growth associated product (U g^{-1}), β is the coefficient for non-growth associated product (U g^{-1} day^{-1}) and *X* is the concentration of biomass (g L^{-1}).

3.10.2.1 Derivation of Luedeking Piret (LP) Model Incorporating Logistic Growth Model

The general equation of LP model (Equation 3.14) is a function of concentration and production rate of biomass. Since the LP model is incorporated with logistic model, variable *dX/dt* in Equation 3.7 and *X* in Equation 3.13 are substituted into Equation 3.11 to give:

$$\frac{dP}{dt} = \alpha\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right] + \beta\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-(e^{\mu_{max}\,t})\right)}\right]$$

Rearrange equation:

$$dP = \alpha\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right]dt + \beta\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-(e^{\mu_{max}\,t})\right)}\right]dt \qquad \text{(Equation 3.15)}$$

Integrate Equation 3.15 with initial conditions, $t = 0$ ($X = X_0$ and $P = P_0$):

$$\int_{P_0}^{P} dP = \int_0^t \alpha\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right]dt + \int_0^t \beta\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})}\right]dt$$

$$(P)_{P_0}^{P} = \alpha \left[\frac{X_0(e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} \right]_0^t + \beta \left[\frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, t})\right)}{\mu_{max}} \right]_0^t$$

$$P - P_0 = \alpha \left[\frac{X_0(e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - \frac{X_0(e^{\mu_{max}\, 0})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, 0})} \right] + \beta \left[\frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, t})\right)}{\mu_{max}} - \frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, 0})\right)}{\mu_{max}} \right]$$

$$P - P_0 = \alpha \left[\frac{X_0(e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, t})\right)}{\mu_{max}} - \frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(1)\right)}{\mu_{max}} \right]$$

$$P - P_0 = \alpha \left[\frac{X_0(e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\frac{X_{max}\, \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, t})\right)}{\mu_{max}} - \frac{X_{max}\, \ln(X_{max})}{\mu_{max}} \right]$$

$$P - P_0 = \alpha \left[\frac{X_0(e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\left(\frac{X_{max}}{\mu_{max}}\right) \ln\left(X_{max} - X_0 + X_0(e^{\mu_{max}\, t})\right) - \ln(X_{max}) \right]$$

$$P - P_0 = \alpha \left[\frac{X_0 (e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\left(\frac{X_{max}}{\mu_{max}}\right) \ln \left(\frac{X_{max} - X_0 + X_0 (e^{\mu_{max}\, t})}{X_{max}} \right) \right]$$

Simplify equation:

$$P - P_0 = \alpha \left[\frac{X_0 (e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\left(\frac{X_{max}}{\mu_{max}}\right) \ln \left(1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t}) \right) \right]$$

The final solution for LP model incorporated logistic growth model is written as:

$$P - P_0 = \alpha \left[\frac{X_0 (e^{\mu_{max}\, t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t})} - X_0 \right] + \beta \left[\left(\frac{X_{max}}{\mu_{max}}\right) \ln \left(1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\, t}) \right) \right] \quad \text{(Equation 3.16)}$$

3.10.3 Kinetics of Glucose Utilization

From the experiment, glucose anhydrous (Systerm) was used as primary carbon source for the batch fermentation. In general, glucose is needed to form cell components, metabolic products and cell maintenance. Equation 3.17 describes the rate of glucose utilization:

$$-\frac{dS}{dt} = \frac{1}{Y_G}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{ps}}\left(\frac{dP}{dt}\right) + m_s(X) \quad \text{(Equation 3.17)}$$

where *dS/dt* is the rate of utilization of glucose (g L^{-1} day^{-1}), *dX/dt* is the rate of biomass production (g L^{-1} day^{-1}), *dP/dt* is the rate of product formation (g L^{-1} day^{-1}), Y_G is coefficient of biomass yield over substrate (g g^{-1}), Y_{PS} is coefficient of product yield over substrate (g g^{-1}), m_S is cell maintenance coefficient (g g^{-1} day^{-1}) and *X* is biomass concentration (g L^{-1}).

Based on the fermentation profile, laccase secretion was increased during the depletion of glucose. In this case, laccase is not a metabolic product and the enzyme is produced through a separate pathway. Thus, glucose is not directly utilized for laccase formation. The equation for glucose utilization can be written without product formation term:

$$-\frac{dS}{dt} = \frac{1}{Y_G}\left(\frac{dX}{dt}\right) + m_s(X) \quad \text{(Equation 3.18)}$$

3.10.3.1 Derivation of Modified Luedeking Piret (LP) Model Incorporating Logistic Growth Model

Hybrid Luedeking-Piret-logistic model was used in the kinetic study of glucose utilization in fungal laccase production. By adding new variables, *m* and *n*, a MLP equation can be written as:

$$-\frac{dS}{dt} = m\left(\frac{dX}{dt}\right) + n(X) \quad \text{(Equation 3.19)}$$

where *m* is the amount glucose required for cell growth and *n* is the amount of glucose for non growth activity. By comparing with Equation 3.18, variables *m*

and n are m = $1/Y_G$ and $n = m_S$ respectively. Equation 3.19 is actually a modification of LP equation (Equation 3.14) which has coefficients for growth associated (α) and non-growth associated (β). By incorporating logistic growth model, variables dX/dt in Equation 3.7 and X in Equation 3.13 are substituted into Equation 3.19 to give:

$$-\frac{dS}{dt} = m\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right] + n\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-(e^{\mu_{max}\,t})\right)}\right]$$

Rearrange equation:

$$-dS = m\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right]dt + n\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)\left(1-(e^{\mu_{max}\,t})\right)}\right]dt \qquad \text{(Equation 3.20)}$$

By using initial condition, $t = 0$ ($X = X_0$ and $P = P_0$), integration of Equation 3.20 yields:

$$-\int_{S_0}^{S} dS = \int_0^t m\left[\mu_{max}\left(1-\frac{X}{X_{max}}\right)X\right]dt + \int_0^t n\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})}\right]dt$$

$$-(S)_{S_0}^{S} = m\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})}\right]_0^t + n\left[\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(e^{\mu_{max}\,t})\right)}{\mu_{max}}\right]_0^t$$

$$-(S-S_0) = m\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})} - \frac{X_0(e^{\mu_{max}\,0})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,0})}\right] + n\left[\frac{\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(e^{\mu_{max}\,t})\right)}{\mu_{max}} - }{\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(e^{\mu_{max}\,0})\right)}{\mu_{max}}}\right]$$

$$S-S_0 = -m\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})} - X_0\right] - n\left[\frac{\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(e^{\mu_{max}\,t})\right)}{\mu_{max}} - }{\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(1)\right)}{\mu_{max}}}\right]$$

$$S-S_0 = -m\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}t})} - X_0\right] - n\left[\frac{X_{max}\,\ln\left(X_{max}-X_0+X_0(e^{\mu_{max}\,t})\right)}{\mu_{max}} - \frac{X_{max}\,\ln(X_{max})}{\mu_{max}}\right]$$

$$S-S_0 = -m\left[\frac{X_0(e^{\mu_{max}\,t})}{1-\left(\frac{X_0}{X_{max}}\right)(1-e^{\mu_{max}\,t})} - X_0\right] - n\left[\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left(\frac{X_{max}-X_0+X_0(e^{\mu_{max}\,t})}{X_{max}}\right)\right]$$

(Equation 3.21)

Simplify and substitute $m = \frac{1}{Y_G}$ and $n = m_S$ into Equation 3.21:

$$S - S_0 = -\frac{1}{Y_G}\left[\frac{X_0(e^{\mu_{max}\,t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\,t})} - X_0\right] - m_S\left[\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left(1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\,t})\right)\right]$$

The final solution for hybrid Luedeking-Piret-logistic model is written as:

$$S - S_0 = -\frac{1}{Y_G}\left[\frac{X_0(e^{\mu_{max}\,t})}{1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\,t})} - X_0\right] - m_S\left[\left(\frac{X_{max}}{\mu_{max}}\right)\ln\left(1 - \left(\frac{X_0}{X_{max}}\right)(1 - e^{\mu_{max}\,t})\right)\right] \quad \text{(Equation 3.22)}$$

3.11 Partial Purification of Laccase

A total of 1.0 L of fermentation medium from the STR cultivation was harvested after 14 days of cultivation. The medium was filtered using Whatman No. 42 filter paper (120 mm diameter) in order to remove fungal cell debris. The filtrate was then centrifuged using Sorvall Centrifuge at 10,000 *g* for 30 minutes at temperature of 4 to 6 °C. After centrifugation, the supernatant was stored at -20 °C prior to purification process.

3.11.1 Protein Fractionation using Ammonium Sulphate Precipitation

$(NH_4)_2SO_4$ salt was slowly added into the sample until 20 % saturation level. The mixture was gently stirred using magnetic bar for 1 hour before centrifugation at 10,000 *g* for 20 minutes. Precipitate was collected and washed using sodium

citrate buffer pH 4.80 in order to remove supernatant. Then, the precipitate was dissolved in 5 mL of sodium citrate buffer (pH 4.80) for laccase activity and protein concentration determinations. The remaining supernatant was used for a series of saturation level *viz* 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 % using the same method. All precipitation was carried out at temperature 25 ± 2 °C. Enzyme activity, protein content and specific activity of each fraction were determined. One of the advantages using ammonium sulfate fractionation is the precipitated protein show better stabilization compared to other techniques. Precipitated protein from the ammonium sulphate method is useful for keeping the protein sample during the long purification process. The high amount of salt prevents the protein sample from the proteolysis and microbial degradation.

3.11.1.1 Calculation of Ammonium Sulfate Precipitation

The amount of ammonium sulfate added in a solution depends on the working temperature. The total amount of ammonium sulfate salt added was based on the equation below:

$$(NH_4)_2SO_4 \text{ weight (g) in 1 L solution } = \frac{G_{sat} \times (S_2 - S_1)}{100 - P_{Salt} \times S_2} \qquad \text{(Equation 3.23)}$$

where G_{sat} is amount of salt contained in 1 L of 100% saturated solution (g); S_1 is initial concentration of saturated solution (%) and S_2 is concentration of saturated solution (%). The salt saturation constant P_{Salt} can be determined as follows:

$$P_{Salt} = \frac{\text{Specific volume} \times G_{sat}}{1000} \qquad \text{(Equation 3.24)}$$

Density and molarity of ammonium sulphate solution at different temperature is summarized (Appendix F).

3.11.1.2 Percentage recovery of laccase and protein

The percentage recovery of laccase and protein in the protein fractionation using ammonium sulfate precipitation method were calculated as follows:

$$\text{Laccase recovery (\%)} = \frac{\text{Precipitated laccase } (\text{U L}^{-1})}{\text{Crude sample laccase } (\text{U L}^{-1})} \times 100\% \quad \text{(Equation 3.25)}$$

$$\text{Protein recovery (\%)} = \frac{\text{Precipitated protein } (\text{g L}^{-1})}{\text{Crude sample protein } (\text{g L}^{-1})} \times 100\% \quad \text{(Equation 3.26)}$$

The precipitated samples were redissolved in the sodium citrate buffer pH 4.8 before furthered assay for laccase activity and protein concentration of the precipitated sample.

3.11.1.3 Purification factor

In order to determine the purity of each fraction from the protein fractionation using ammonium sulfate precipitation, purification factor was calculated as follows:

$$\text{Purification factor} = \frac{\text{Specific activity fraction } (\text{U g}^{-1})}{\text{Specific activity of crude sample } (\text{U g}^{-1})} \quad \text{(Equation 3.27)}$$

3.11.2 Gel Filtration using Sephadex

Gel filtration was carried out using two types of Sephadex i.e. G-25 and G-75 from GE Healthcare. Sephadex or known as dextran gel is a polysaccharide which contain glucose residues joined by α-1,6-glucosidic linkages (Fischer and Work, 1980). Sephadex dissolve readily in water, cross-linked to form a gel where the polysaccharide chains form a three-dimensional network.

Sephadex in the gel form is chemically stable. Sephadex G-75 is still intact after 6 months in 0.02 M HCl (Cruft, 1961). Meanwhile Sephadex G-25 was found stable after 2 months in 0.25 M of NaOH at 60 °C (Fischer and Work, 1980). Swelling and drying of Sephadex gel beads is a reversible process. The material retains its chromatographic capability after repeated drying and reswelling. Another important characteristic of Sephadex is it is also stable in alkaline solutions which makes it is possible to remove contaminants such as denatured protein or lipids that precipitated on the gel by treatment with strong alkali. The technical data for Sephadex gel matrix is summarized in Appendix G.

In this study, Sephadex G25 was used in desalting column due to low fractionation range (M_r) of 1 to 5 kDa which is suitable for group separation of globular protein molecules. The medium is excellent for the removal of low molecular mass impurities such as salts, sugars, etc. Sephadex G-75 was used for laccase separation as it has fractionation range (*Mr*) of 3 to 80 kDa which is a suitable range for laccase with molecular mass approximately ranging between 60 to 80 kDa (Garcia *et al.*, 2006).

For chromatography column, a standard C10/20 column (length: 20.0 cm, internal diameter: 1.0 cm) from GE Healthcare was used. The chromatography experiment was performed based on the parameters summarized in Table 3.6. A schematic set up for column chromatography is shown in Figure 3.2. For

determination of void volume, Dextran Blue (Sigma) was used as it has high molecular weight about 2000 kDa. Void volume calculation can be determined using Equation 3.28:

$$V_0 = V_T - V_M \quad \text{(Equation 3.28)}$$

where V_0 is void volume (mL), V_T is total column volume (mL) and V_M is gel matrix volume (mL). Dextran blue will be excluded completely as high molecular mass of the substance prevents it from entering matrix pores. The dilution factor for the amount of sample loading in the gel filtration column can be calculated as:

$$DF = \frac{V_E}{V_{SL}} \quad \text{(Equation 3.29)}$$

where DF is dilution factor, V_E is total volume of eluted sample (mL) and V_{SL} is total volume of sample loaded (mL).

Table 3.6: Parameters for gel chromatography experiment

Parameter	**Value**	
	Sephadex G-25	**Sephadex G-75**
Sample loading (L)	0.0005	0.0005
Elution fraction (L)	0.0005	0.0005
Linear flow rate (L min^{-1})	0.001	0.001
Volumetric flow rate (cm h^{-1})	76.36	76.36
Temperature (°C)	25 ± 2	25 ± 2
Elution buffer	Sodium citrate (50 mM, pH 4.80)	Sodium citrate (50 mM, pH 4.80)

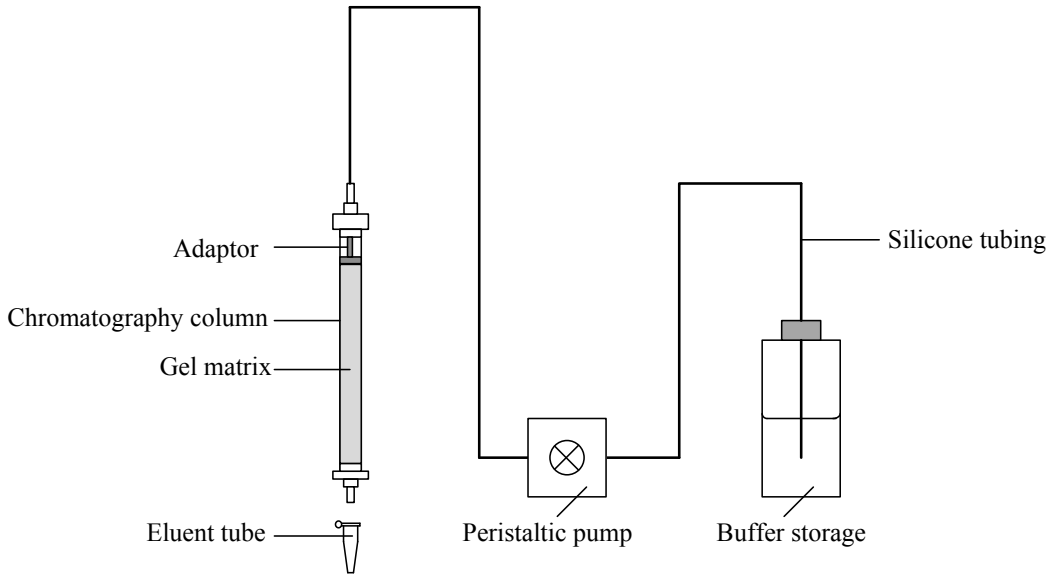


Figure 3.2: Schematic diagram of gel chromatography column set up

3.12 Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on partially purified sample from the gel filtration experiment. Standard preparation method for SDS PAGE experiment is according to Laemmli (1970) (Appendix H). Experiment was carried out using commercial gel electrophoresis kit Mini-Protean 3 Cell from Bio Rad. The kit contained glass plates, casting apparatus, gel comb, gel cassette assembly, electrode assembly, inner chamber, mini tank and Lid. The kit was assembled according to the instruction manual from Bio Rad.

The resolving and stacking gel formulation was prepared at 12 % gel strength (Appendix E). The resolving gel was prepared by adding 1.7 mL of ultrapure water, 2.0 mL of 30 % Acrylamide/Bis, 1.25 mL of gel buffer (1.5 M Tris-HCl, pH 8.8) and 0.05 mL of 10 % (w/v) SDS. The mixture was degassed using vacuum pump for 8 to 10 minutes. After degassing, gel mixture was added with 25 µL of 10 % APS and 2.5 µL of TEMED. The gel mixture was swirled gently for a few seconds and immediately poured into the assembled glass plate

until gel surface reaches suitable height. The top of the gel surface was covered with a 1 cm thick layer of ultrapure water and the gel was allowed to polymerize for 40 to 60 minutes at room temperature. The top layer of ultrapure water was discarded prior to the addition of stacking gel.

For the preparation of stacking gel, the same preparation in resolving gel was used except for the volume of TEMED which was 5 µL. The stacking gel was added after the resolving gel was completely polymerized. A gel comb was carefully inserted into top of stacking gel and was left at room temperature for 40 to 60 minutes for complete polymerization. All experiments involving gel preparation have to be done carefully in order to avoid bubble formation inside the gel.

Protein sample was diluted with sample buffer (SDS reducing buffer) with the proportion of protein sample to buffer at 1 : 2 and incubated in a hot waterbath (95 °C) for 5 minutes. By using the same method for protein sample preparation, a wide range of protein marker (Sigma) was prepared as standard according to supplier's instruction.

The mini tank and inner chamber was assembled before removing the gel comb from the stacking gel. 9 µL of protein sample and protein standard was added into the stacking gel and the lid was placed on top of the mini tank. The mini tank was connected into the power supply with current voltage 170 V for about 40 to 50 minutes to complete gel separation.

Protein electrophoresis band was stained using silver staining method by Heukeshoven and Dernick (1985) (Appendix I). The molecular weight of protein sample was compared with protein standards. All buffers and reagents preparation for SDS PAGE and silver staining method are prepared using ultrapure water.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Improvement of Liquid Medium for Laccase Production

Experiments were performed in shake flasks with the effects of complex nutrients i.e. yeast extract, malt extract and peptone on laccase production were analyzed using full factorial design (FFD). All fermentation runs were done in random order according to design matrix generated by the software. The overall response for the liquid medium screening is summarized in Table 4.1.

Table 4.1: Responses for the FFD experiments on liquid medium improvement

Run	Nutrients (g L^{-1})			Response (U L^{-1})
	Yeast (*A*)	Malt (*B*)	Peptone (*C*)	
1	0	0	0	0.0
2	0	2	0	39.6
3	0	0	0	0.0
4	2	0	0	0.0
5	2	2	2	35.8
6	2	2	2	37.4
7	0	2	2	34.2
8	0	0	2	0.0
9	0	2	2	37.5
10	2	2	0	40.1
11	0	0	0	0.0
12	0	2	0	41.2
13	2	2	0	35.7
14	2	0	2	0.0
15	0	2	0	37.1
16	2	0	2	0.0
17	2	2	2	34.6
18	0	2	2	40.1
19	2	0	0	0.0
20	2	0	0	0.0
21	2	2	0	34.5
22	0	0	2	0.0
23	2	0	2	0.0
24	0	0	2	0.0

The highest productivity was achieved when malt extract (*B*) was used as sole complex nutrient with an average enzyme productivity of 39.3 ± 2.1 U L^{-1}.

There was very little enzyme production when either yeast extract (*A*) or peptone (*C*) was added into liquid medium as sole complex nutrient. The average of enzyme productivity for combination of *B*-*A* and *B*-*C* was 36.8 ± 2.9 and 37.3 ± 2.1 U L^{-1}, respectively. However the combination of *A*-*C* showed no laccase production. The combination of all three complex nutrients *A*-*B*-*C* yielded an average productivity of 35.9 ± 1.4 U L^{-1}.

4.1.1 Analysis of FFD Experiments on Liquid Medium Improvement

Statistical analysis of experimental data in Table 4.1 was done by using analysis of variance (ANOVA) for determination of significant factors (Table 4.2). The responses obtained from FFD experiment were analyzed in order to determine significant level of the effect of yeast extract, malt extract and peptone for maximum laccase production.

Table 4.2: First order ANOVA analysis for the effect of yeast extract, malt extract and peptone on liquid medium improvement

Descriptive Statistic					
R^2	0.9932				
R^2 adjusted	0.9922				
S_{Error}	1.6901				
PRESS	82.2648				
ANOVA					
Term	**DoF**	**Adj SS**	**Adj MS**	***F***	***P***
Yeast (*A*)	1	5.61	5.61	1.96	0.177
Malt (*B*)	1	8,355.20	8,355.20	2,925.06	0.000
Peptone (*C*)	1	3.08	3.08	1.08	0.311
Lack of fit	4	9.77	2.44	0.83	0.528
Pure error	16	47.36	2.96		
Total	23	8,421.02			

R^2 = Correlation coefficient; S_{Error} = Standard error regression; PRESS = Prediction sum of squares; DoF = Degrees of freedom; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean of squares; *F*: F-statistic; *P*: P-statistic

From the analysis of variance in Table 4.2, the effect of main factor yeast extract and peptone were not significant ($P > 0.05$) in improving the extracellular laccase production. The effect of malt extract in the liquid medium was found to be significant ($P < 0.05$). The experimental data was fitted to a first order model terms which only based on main factors. Descriptive statistic indicated the R^2 and R^2 *adjusted* value of 0.9932 and 0.9922 respectively which suggested the experimental data is in good correlation with the model. From the analysis, lack of fit test was not significant with $P = 0.528$ was less than selected α level ($p = 0.05$). When lack of fit test was not significant, the first order model accurately fit the data. The lack of fit was assessed because of the factorial experiment was reduced from default order to first order. The advantage of using reduced model is the mathematical equation used to describe the overall experimental data is simpler. A reduced model from full model analysis can generate less the number of experiments by modelling only significant term or by running fractional factorial design (Montgomery, 2001; Tinoco *et al.*, 2011).

4.1.2 Residual Analysis of FFD Experiments on Liquid Medium Improvement

Residual analysis was carried out using the standardized residual (Figure 4.1). The normal probability plot (Figure 4.1 a) indicated there was one potential outlier data point in the plot which was indicated by blue point. To confirm this, an outlier test using Grubb Test was performed. The test indicated that there was no outlier detected in the data. By using the standardized residuals (d_i) which can be defined as:

$$d_i = \frac{r_i}{\sigma} \qquad \text{(Equation 4.1)}$$

where r_i is a actual residual observation and σ is standard deviation. The standardized residual, $d_i \geq 3$ is categorized as potential outlier (Montgomery, 2001). The actual residual observation (r_i) in Equation 4.1 can be written as follows:

$$r_i = y_{i\ observe} - y_{i\ predicted} \qquad \text{(Equation 4.2)}$$

where y_i is the response observation (i = 1,2,3…,n). An over-fitting result is indicated by the large residual value. From the experiment, the standardized residual value was less than ± 3 and thus not a potential outlier. All the data was distributed reasonably well along the blue line which indicated that the data followed a normal distribution pattern (Figure 4.1 a).

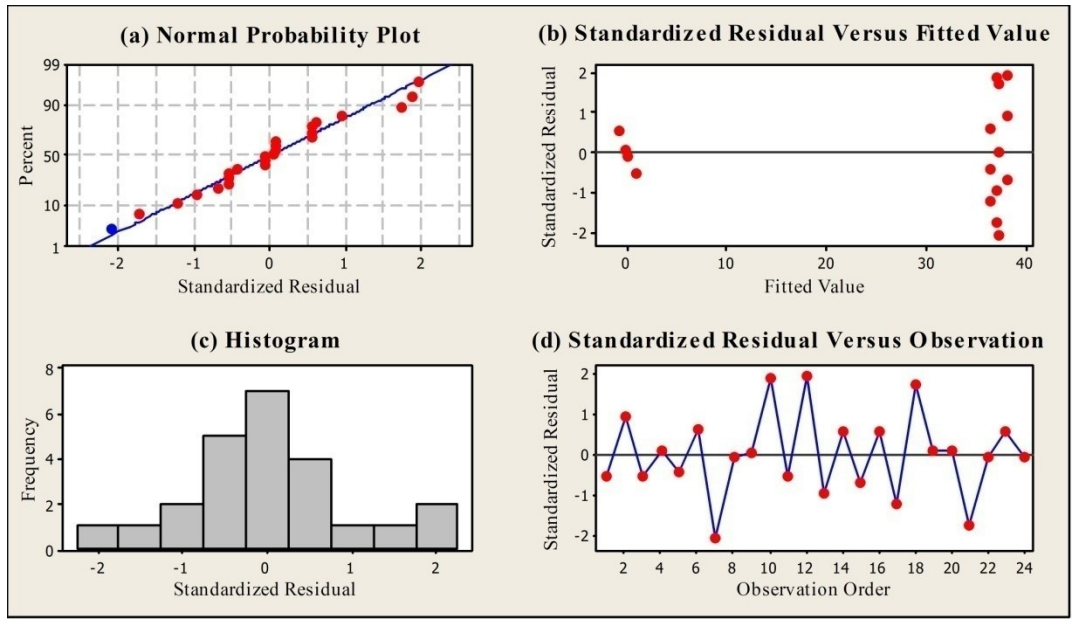


Figure 4.1: Residual plot analysis for FFD Experiment in liquid medium improvement

In the residuals versus fitted values plot (Figure 4.1 b), the variance between the observed and fitted value was rather large. The variance also showed an increase of residual when predicted response (fitted value) increases. This may indicate that as a response from the experiments is getting higher, the possibility of error also increases.

However the histogram of residuals (Figure 4.1 c) showed that experimental data follow a general normal distribution trend with a short tail observed at both side of the distribution. The peak of histogram was observed to be on zero standardized residual. The high frequency of zero standardized residual in histogram was due to a large number fermentation runs with zero laccase production. The effect of experimental order on the response obtained did not show any unusual patterns (Figure 4.1 d). Thus, the effect of fermentation runs did not influence the overall response since the data points were equally distributed above and below the standardized line.

4.1.3 Main Effect Plot of FFD Experiments on Liquid Medium Improvement

The main factors of the screening experiment were studied using the factorial effect plot. From the main effect plot (Figure 4.2), the addition of 2.0 g L^{-1} malt in the medium increases the production of extracellular laccase. This was indicated by the increase in experimental data mean. However the addition of yeast extract and peptone did not show any improvement on the mean.

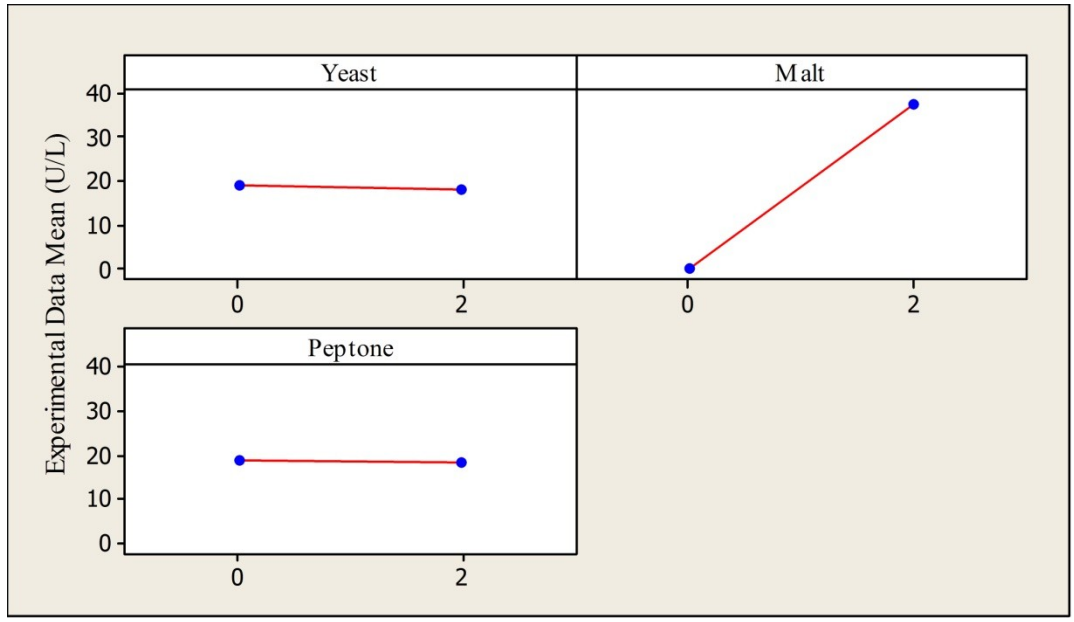


Figure 4.2: Main effect plot for FFD Experiment in liquid medium improvement

Previous study by (Arora and Gill, 2001) indicated the maximum laccase production was obtained when cultivated in medium containing mineral salt-malt extract broth (MSB-MEB). Malt extract is rich in amino acid tryptophan and tyrosine (Arora and Gill, 2001). According to Eggert *et al.* (1996), the main content of malt extract that influence the maximum production of laccase is identified as tryptophan derivative metabolites (3-hydroxy-2-aminobenzote) which acts as a mediator in laccase-catalyzed oxidation reactions.

From the analysis, the addition of malt (2.0 g L^{-1}) significantly influenced the extracellular laccase production. The addition of yeast and peptone did not influence the enzyme production. The slope from the main effect plot of malt was the steepest compared to the main effect plot of yeast extract and peptone.

4.1.4 The Effect of Glucose Concentrations on Liquid Medium Improvement

In Section 4.1, the glucose concentration used was fixed at 25 g L^{-1}. By using the best medium formulation obtained from the main effect plot in Figure 4.2, the effect of 5 and 10 g L^{-1} of glucose were studied. Extracellular laccase

activity after 7 days of fermentation was used as a response. Parameters such as agitation speed and pH were fixed at constant value in order to evaluate the effect of different glucose concentration on enzyme productivity. One way ANOVA analysis (Table 4.3) on the data obtained was performed by Sigma Plot software. The *p* value (0.7472) for glucose concentration effect showed that all the means were equal when α is 0.05. As conclusion the effect of different glucose concentration did not significantly influence laccase production after 7 days. In addition, the differences in the mean values are small to remove the possibility that the difference is due to the sampling error.

Table 4.3: One way ANOVA of glucose concentrations in liquid medium improvement

Descriptive Statistic				
Concentration	**N**	**Mean**	**σ**	**SEM**
25	3	39.3000	2.0664	1.1930
10	3	37.7667	2.9535	1.7052
5	3	38.0333	2.5968	1.4993

ANOVA					
Source	**DoF**	**SS**	**MS**	***F***	***P***
Glucose concentration	2	4.0267	2.0133	0.3060	0.7472
Residual error	6	39.4733	6.5789		
Total	8	43.5000			

N: No of sample; σ: Standard deviation; SEM: Standard error of mean; DoF: Degrees of freedom; SS: Sum of squares; MS: Mean squares; *F*: F-statistic; *P*: P-statistic.

4.1.5 Determination of Best Liquid Medium for Laccase Production

ANOVA test on liquid medium screening experiment (Table 4.2, Section 4.1.1) indicated that the addition of malt extract (2.0 g L^{-1}) significantly enhanced laccase production. However the effect of different glucose concentration (Table 4.3, Section 4.1.4) was insignificant in the extracellular laccase production after 7 days. Therefore, glucose with low concentration was added sufficient for the fungus to perform growth metabolism. The final formulation of liquid medium for

laccase production is summarized in Table 4.4. This medium will be used in all fermentations for laccase production in shake flasks and stirred tank reactor.

Table 4.4: Laccase production medium formulation

Components	Amount added (g L^{-1})
Glucose anhydrous	5.0
Malt extract	2.0
$CaCl_2$	0.1
$CuSO_4.5H_2O$	0.01
K_2HPO_4	0.1
KCl	0.05
KH_2PO_4	2.0
$MgSO_4.7H_2O$	0.5
$(NH_4)_2SO_4$	2.0

4.2 Fermentation in Shake Flasks

By using liquid medium for laccase production (Table 4.4, Section 4.1.5), the time courses of fungal biomass, laccase production and glucose utilization were studied. Profiling experiments in shake flasks were performed in baffled and non-baffled flasks. For the kinetic studies, the cultivations in baffled and non-baffled flasks were carried out until biomass concentration has reached its maximum value.

4.2.1 Theoretical Estimation of Mass Transfer Coefficient (k_La) in Shake Flask

Based on the equation postulated by Henzler and Schedel (1991) in Section 3.4.1, the parameter constants are shown in Table 4.5. Distilled water was used as standard liquid for the k_La calculation in shake flasks. The postulated equation does not account the effect of baffled parameter. Thus, the theoretical k_La values in the baffled flasks were not determined. The theoretical k_La values in shake flask at 90, 170 and 250 rpm are shown in Table 4.6.

Table 4.5: Parameters constant for k_La calculation

Parameters		Value
d	Maximum inner flask diameter	8.5×10^{-2} m
d_0	Shaking diameter	2.5×10^{-2} m
V_L	Filling volume	1.0×10^{-4} m^3
D	Diffusion coefficient of dissolved oxygen in water at 25 °C	2.1×10^{-9} m^2 s^{-1} (Cussler, 1997)
v	Kinematic viscosity at 25 °C	8.7×10^{-7} m^2 s^{-1} (Viswanath *et al.*, 2007)
g	Gravity acceleration	9.81 m s^{-2}

Table 4.6: Theoretical k_La value in shake flasks

Flasks	Agitation (rpm)	k_La (s^{-1})
Non baffled	90	0.0193s
	170	0.0364
	250	0.0535

4.2.2 Fungal Growth Profiles in Baffled and Non-baffled Flasks

The effect of different agitations (90, 170 and 250 rpm) on fungal growth, laccase production and glucose utilization were evaluated in baffled and non-baffled flasks. Sigma Plot 11 software was used to draw the curve profile. According to Bermek *et al.* (2004), baffled flasks have unique design which enable strong convection current upon stirring which improve mixing air intake into the medium. Fungal growth and their standard deviation in Figure 4.3 indicated the cultivation in baffled flasks significantly increased biomass production at 90 and 170 rpm. At 250 rpm, the growth profile indicated the overlapping curves between baffled and non-baffled cultivations. The low maximum biomass concentration at high agitation indicating that fungal growth was impaired at higher agitation such as 250 rpm. Henzler and Schedel (1991) suggested that the use of baffled flasks at high agitation rate does not offer marked advantages over the non-baffled flasks as oxygen transfer coefficient k_La in baffled flasks are not significant greater than the values in the non-baffled ones.

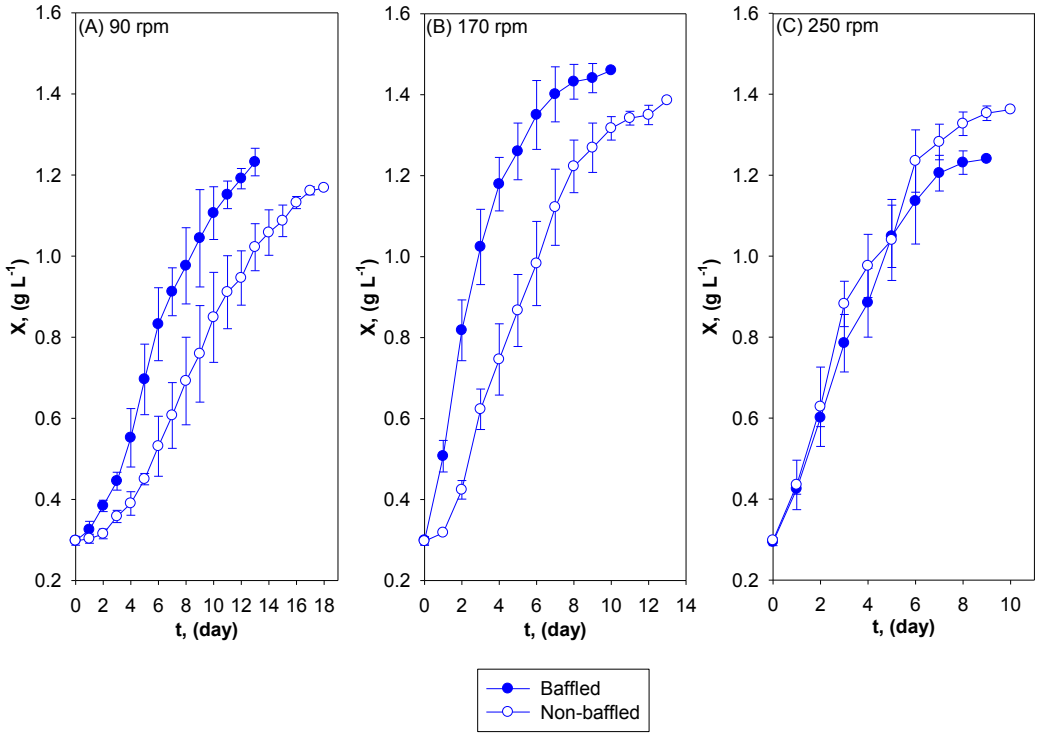


Figure 4.3: Growth profiles in baffled and non-baffled flasks; (A) 90 rpm, (B) 170 rpm and (C) 250 rpm (Error bar indicates standard deviation of 3 replicate experiments)

It was observed that at high agitation rates, some of the cells were attached to the wall of the flasks above the liquid level due to the splashing of liquid medium at high agitation rates which sometimes could be observed in the baffled flasks cultivation (Henzler and Schedel, 1991; Bermek *et al.*, 2004). According to Büchs *et al.* (2001) baffles may disturb the circulating movement of the liquid and causing the system to be out of phase which caused excessive wall growth above the liquid level. This can lead to a problem with the sterility and gas permeability of the cotton plug of the flasks (Büchs, 2001). The maximum biomass concentration was achieved earlier in the baffled flasks compared to the non-baffled flasks. The average cultivation time for fungal growth to reach its maximum biomass concentration was relatively lowered in the baffled cultivations

at 90 and 170 rpm. However an average of cultivation time for fungal growth to reach its maximum biomass concentration at 250 rpm showed same cultivation time based on the standard deviation of the replicates in baffled and non-baffled flasks.

4.2.3 Glucose Utilization Profiles in Baffled and Non-baffled Flasks

Glucose utilization and their standard deviation in Figure 4.4 showed apparent increased between baffled and non-baffled flasks at 90 and 170 rpm. However glucose utilization at 250 rpm for baffled and non-baffled cultivations exhibited no significance difference. The rapid utilization of glucose was observed approximately with the exponential growing phase of fungal cell. The complete exhaustion of glucose was achieved earlier in baffled flasks at 90 to 170 rpm compared to the cultivation at 250 rpm as there was no difference in glucose utilization profile between baffled and non-baffled flasks. This was not observed in fungal cultivations at 90 and 170 rpm as the used of baffled flasks at these agitation speeds facilitated the mass transfer of nutrients and dissolved oxygen content (Buchs 2001).

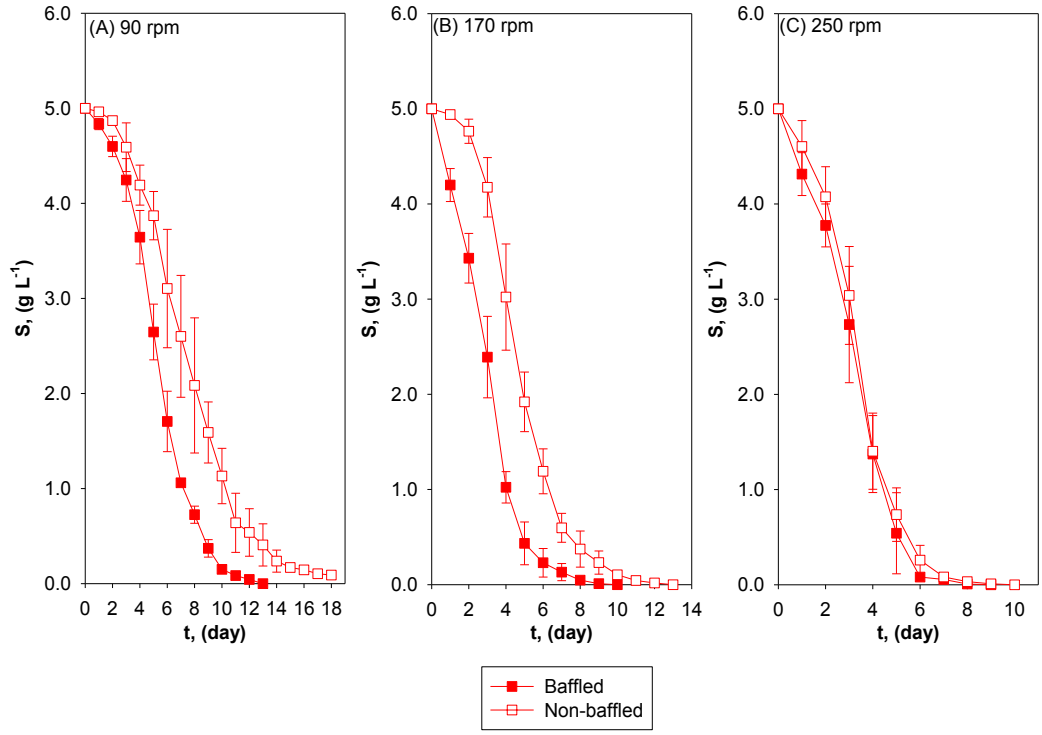


Figure 4.4: Glucose utilization profiles in baffled and non-baffled flasks; (A) 90 rpm, (B) 170 rpm and (C) 250 rpm (Error bar indicates standard deviation of 3 replicate experiments)

4.2.4 Laccase Production Profiles in Baffled and Non-baffled Flasks

Laccase production and their standard deviations (Figure 4.5) indicate apparent increased between baffled and non-baffled flasks at 90 and 170 rpm. In contrast, laccase production in baffled flasks at 250 rpm was slightly decreased compared to the non-baffled flasks. This could be due to the disadvantage of using baffled flasks at high agitation speed such as 250 rpm. There was a delay in the start of laccase production for the non-baffled flasks compared to the baffled flasks.

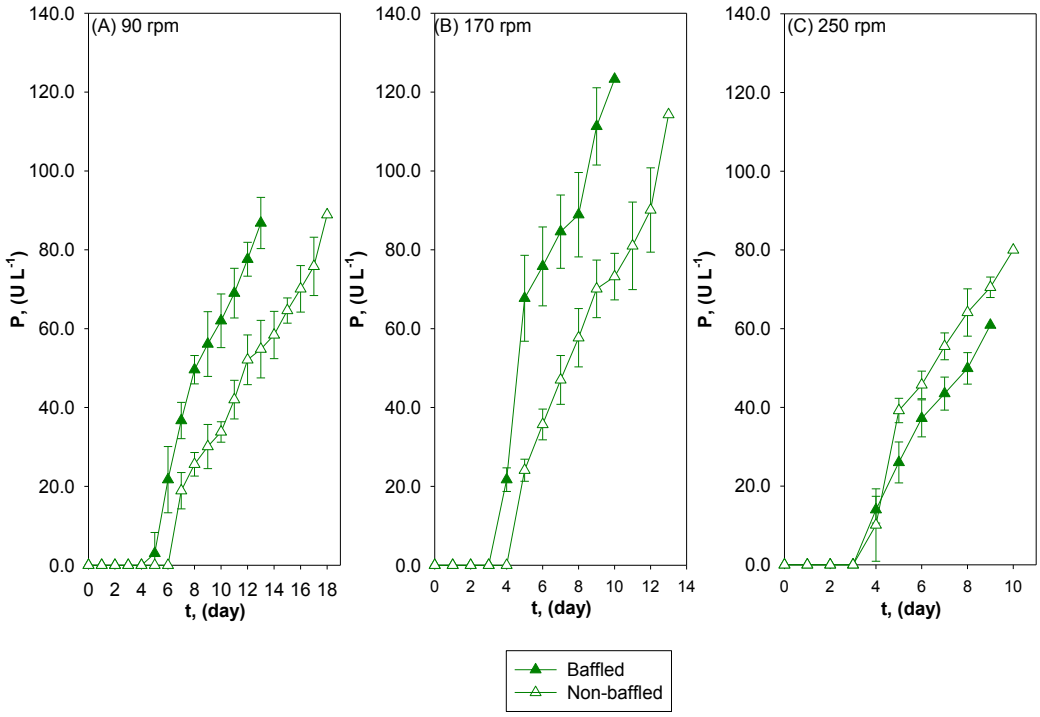


Figure 4.5: Laccase production profile in baffled and non-baffled flasks; (A) 90 rpm, (B) 170 rpm and (C) 250 rpm (Error bar indicates standard deviation of 3 replicate experiments)

At 90 rpm, the start of laccase production in baffled flasks was observed earlier than the non-baffled flasks on the fourth and sixth day respectively. Similar observation was also indicated at 170 rpm with the start of laccase production in baffled and non-baffled flasks on the third and fourth day respectively. However, the start of laccase production in baffled and non-baffled flasks at 250 rpm was observed on the same cultivation day which was on third day.

4.3 Kinetic Parameters Estimation in Baffled and Non-baffled Flasks

Experiments were carried out in batch fermentation. Kinetic studies on laccase production in baffled and non-baffled flasks were performed using agitation speeds of 90, 170 and 250 rpm. By solving the differential equations, the kinetic parameters from the equation were calculated using non linear regression

method. Polymath 6.0 software was used to solve the non-linear equations in order to obtain these parameters. The kinetic parameters describing the rate of biomass formation (*dX/dt*), the rate of glucose utilization (*dS/dt*) and the rate of laccase production (*dP/dt*) and are shown for the baffled flasks (Table 4.7) and non-baffled flasks (Table 4.8).

Table 4.7: Kinetic parameters of fungal growth, glucose utilization and laccase production in baffled flasks

Kinetic Parameters	Agitation		
	90 rpm	170 rpm	250 rpm
Fungal Growth			
X_0 (g L^{-1})	0.298(±0.028)	0.299(±0.024)	0.294(±0.022)
X_{max} (g L^{-1})	1.232(±0.083)	1.454(±0.079)	1.244(±0.042)
μ_{max} (day^{-1})	0.306(±0.087)	0.722(±0.179)	0.568(±0.183)
Glucose Utilization			
Y_G (g g^{-1})	0.177(±0.035)	0.245(±0.036)	0.188(±0.017)
m_S (g g^{-1} day^{-1})	0.039(±0.020)	0.041(±0.015)	0.048(±0.016)
Laccase Production			
α (U g^{-1})	-27.022(±12.274)	-9.133(±2.562)	-15.110(±8.754)
β (U g^{-1} day^{-1})	11.041(±1.193)	12.817(±2.332)	9.712(±2.634)

(± 95% confidence interval)

Table 4.8: Kinetic parameters of fungal growth, glucose utilization and laccase production in non-baffled flasks

Kinetic Parameters	Agitation		
	90 rpm	170 rpm	250 rpm
Fungal Growth			
X_0 (g L^{-1})	0.297(±0.008)	0.297(±0.025)	0.298(±0.019)
X_{max} (g L^{-1})	1.150(±0.048)	1.353(±0.072)	1.361(±0.035)
μ_{max} (day^{-1})	0.206(±0.067)	0.393(±0.080)	0.567(±0.158)
Glucose Utilization			
Y_G (g g^{-1})	0.164(±0.018)	0.215(±0.029)	0.214(±0.015)
m_S (g g^{-1} day^{-1})	0.047(±0.011)	0.043(±0.020)	0.039(±0.028)
Laccase Production			
α (U g^{-1})	-31.520(±23.817)	-9.235(±5.058)	-9.243(± 2.988)
β (U g^{-1} day^{-1})	8.585(±2.326)	9.690(±2.364)	9.894(±1.331)

(± 95% confidence interval)

4.3.1 Kinetics of Fungal Growth in Baffled and Non-baffled Flasks

The effect of baffled and non-baffled cultivations on fungal growth kinetics is evaluated in the form of multiple mean comparisons (Figure 4.6). It is obvious that increase in agitation speeds adversely influenced the fungal growth production in submerged fermentation (Cui *et al.*, 1997; Amanullah *et al.*, 2000; Li *et al.*, 2000). From the observation, the effect of baffled and non-baffled on maximum specific growth rate (μ_{max}) showed insignificant difference at 90 and 250 rpm. However μ_{max} value at 170 rpm showed significant increase in the baffled flasks compared to non-baffled flasks. At different agitations in baffled cultivations showed significant increase between 90 and 170 rpm but insignificant differences observed between 90 and 250 rpm. In the non-baffled flasks, the μ_{max} value was increased significantly with agitation increase from 90 to 170 rpm. This was accompanied by significant increased between 90 and 250 rpm. Comparisons between 170 and 250 rpm indicated insignificant differences in the μ_{max} value. Higher agitation speeds significantly increased the aggregation mechanism at the early stage of the fermentation process resulted in denser pellets (Kelly *et al.*, 2004). This was observed apparently at 170 rpm in baffled and non-baffled flasks. At higher agitation such as 250 rpm, fungal growth in baffled cultivations was impaired probably due to higher shear conditions.

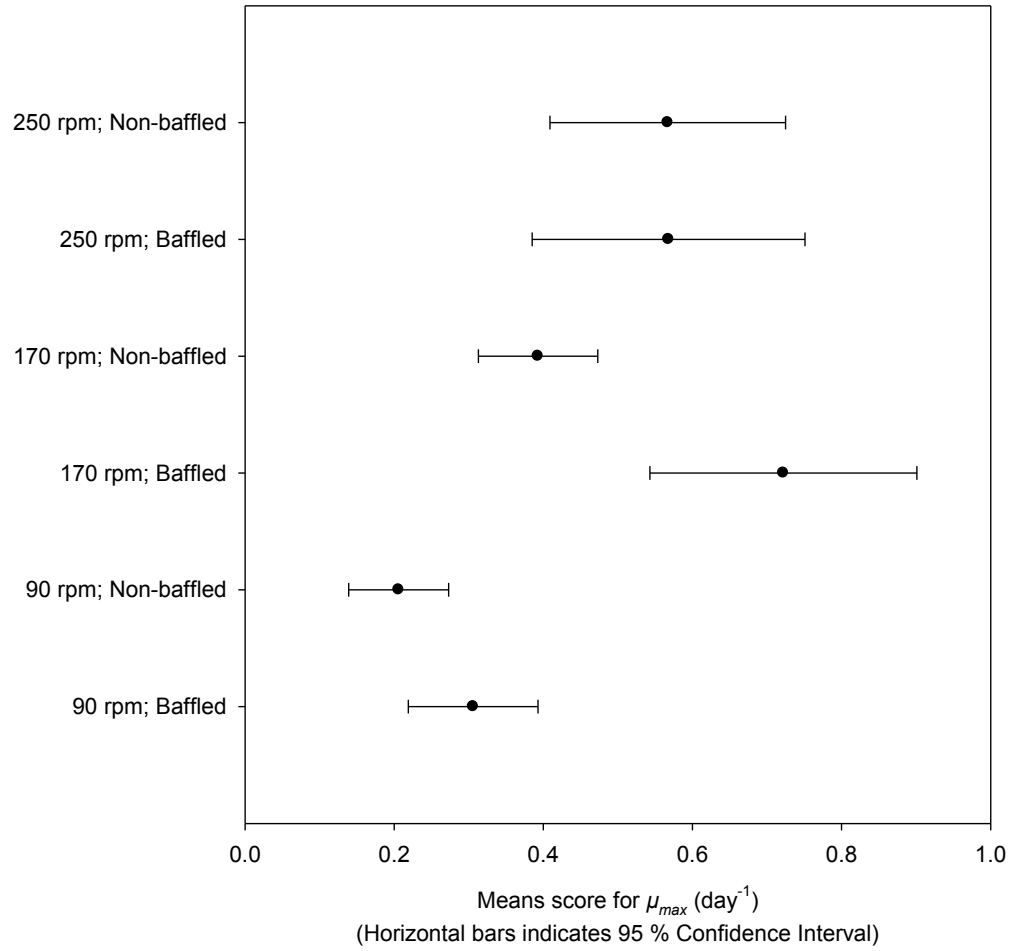


Figure 4.6: Multiple mean comparisons of μ_{max} in baffled and non-baffled flasks

Based on the multiple comparisons of mean (Figure 4.7), maximum biomass concentration (X_{max}) in baffled flasks increased significantly between 90 and 170 rpm. Comparisons between 90 and 250 rpm did not showed significant changes in X_{max} value. However significant decreased in X_{max} value was observed when agitations increased from 170 to 250 rpm. Meanwhile, the X_{max} value in non-baffled cultivations increased significantly when agitation increase from 90 to 170 rpm. Further increase of agitation from 170 to 250 rpm indicated insignificant changes in X_{max} values. At fixed agitation, the effect of baffled and non-baffled flasks on X_{max} showed insignificant differences at 90 and 170 rpm. At 250 rpm, the X_{max} value in non-baffled flasks was significantly increased compared to the

baffled flasks. This phenomenon could be related to the observed excessive biomass wall growth.

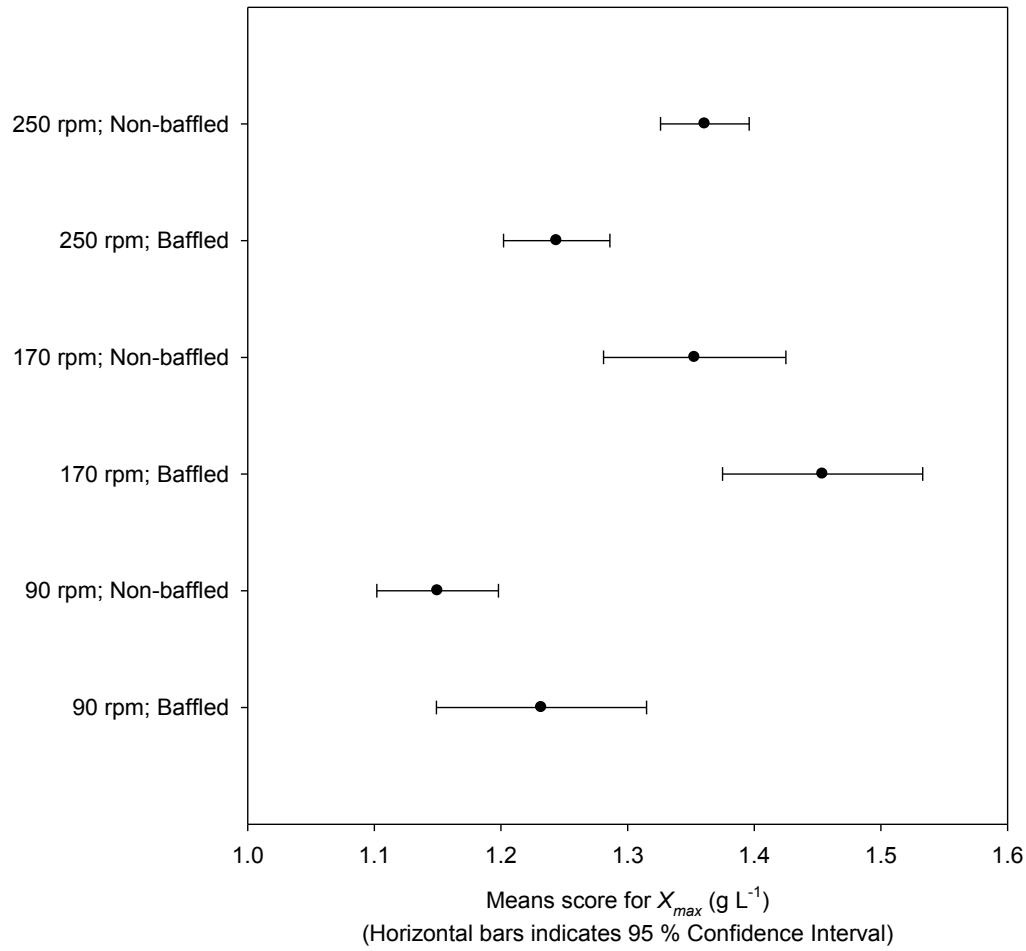


Figure 4.7: Multiple mean comparisons of X_{max} in baffled and non-baffled flasks

According to Figure 4.8, the cultivation time for the fungus to reach its maximum concentrations (t_{Xmax}) was significantly lowered at 90 and 170 rpm in baffled flasks.

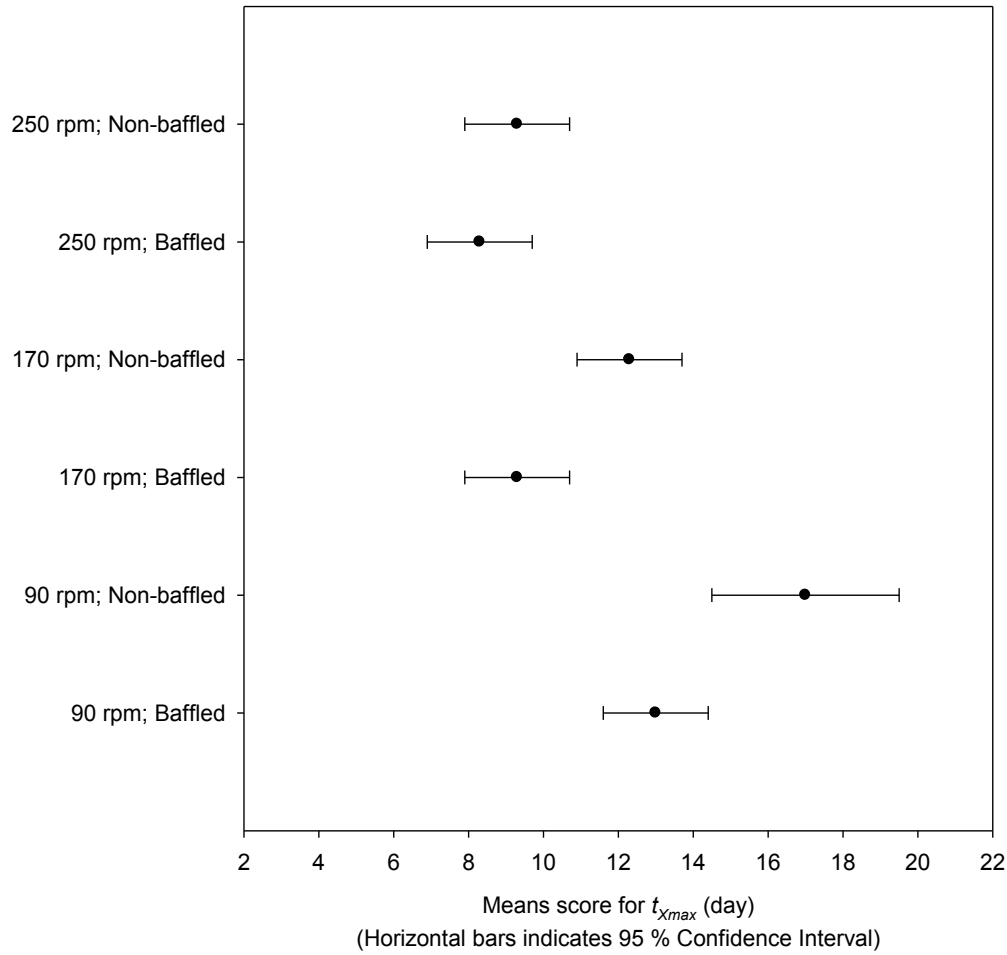


Figure 4.8: Multiple mean comparisons of t_{Xmax} in baffled and non-baffled flasks

However at 250 rpm, the cultivation time for the fungus to reach its maximum concentration indicated insignificant difference between baffled and non-baffled flasks. Shorter cultivation time for fungal to reach maximum concentration can be explain in terms of mass transfer ability. The availability of the nutrients for the fungal population depends on the mass transfer condition in the medium. In the baffled flasks, the fungal populations are segregated resulted in better mass transfer for nutrients and oxygen which could explain the shorter cultivation time for fungal to reach its maximum concentration. In addition the fermentation profiles in shake flasks indicated the time when biomass reached its maximum concentration coincided with depleted glucose concentration in the

medium. Thus, the relatively fast glucose depletion was due to better mass transfer which resulted in shorter cultivation time for fungus to reach its maximum concentration.

4.3.2 Kinetics of Glucose Utilization in Baffled and Non-baffled Flasks

The effect of baffled and non-baffled glucose utilization was performed using the same amount of inoculum and substrate for all shake flasks cultivation. In this case, the over estimation of the effect different flasks geometry and agitations should be avoided. Based on multiple mean comparisons in Figure 4.9, the coefficient of biomass yield over substrate (Y_G) indicated insignificant differences between baffled and non-baffled flasks at fixed agitation. However, Y_G value in baffled flasks showed slight decreased when agitations increased from 170 to 250 rpm. This was accompanied by significant decreased of X_{max} in baffled flasks when agitation increased from 170 to 250 rpm (Figure 4.7 in Section 4.3.1). Significant increased in Y_G value was observed in non-baffled flasks when agitations increased from 90 to 170 rpm.

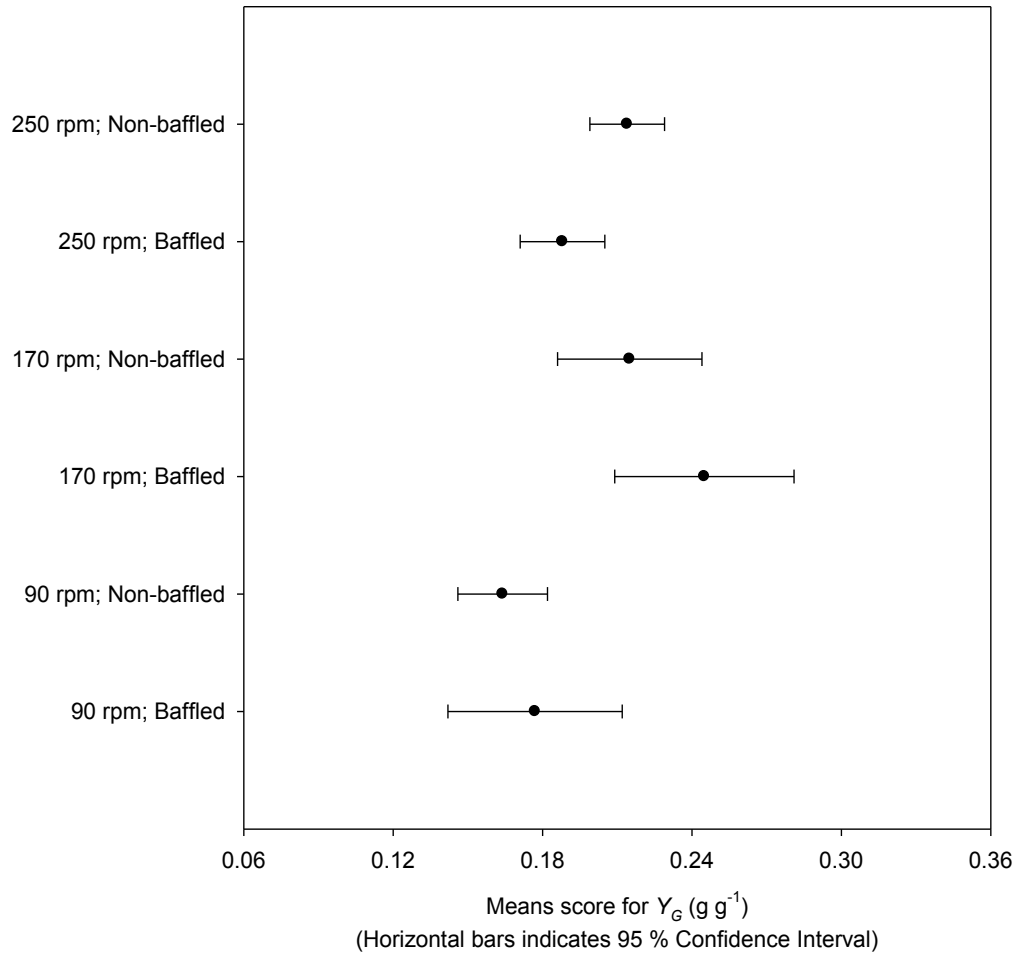


Figure 4.9: Multiple mean comparisons of Y_G in baffled and non-baffled flasks

However, the cell maintenance coefficient (m_S) did not showed significant changes when tested at different flasks geometry and agitations (Figure 4.10). This observation did not correlate with the previous findings in Y_G and X_{max}. As previously reported, the cultures with lower m_S value would allow for higher X_{max} value (Panula-Perälä *et al.*, 2008). In this case, the m_S value in shake flasks was nonlinearly dependent on growth yield, growth rates and endogenous metabolism. Thus, a conceptual model which distinguishes physiological maintenance from other non-growth components should be used in order to describe their relative importance (Bodegom, 2007).

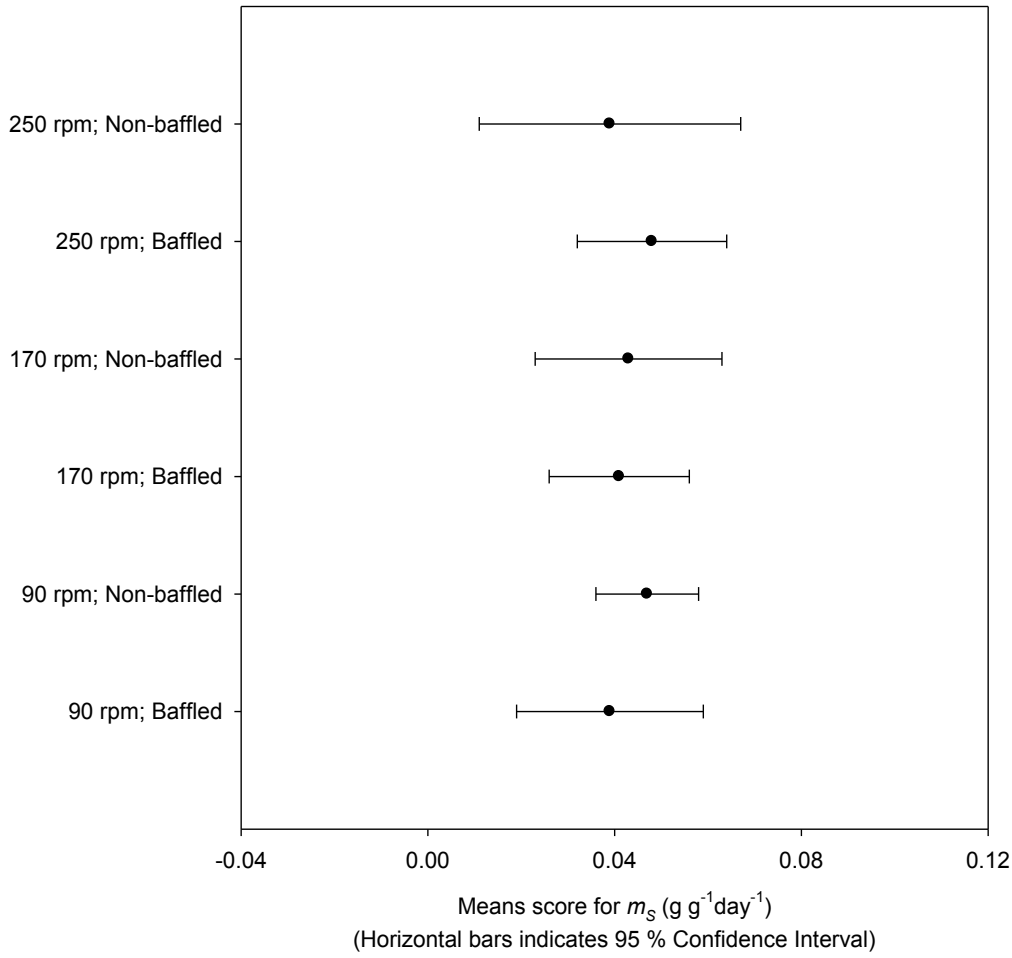


Figure 4.10: Multiple mean comparisons of m_S in baffled and non-baffled flasks

4.3.3 Kinetics of Laccase Production in Baffled and Non-baffled Flasks

The effect of baffled and non-baffled flasks on coefficient for growth associated product (α) was evaluated in the form of multiple mean comparisons (Figure 4.11). With respect to the baffled and non-baffled cultivations and different agitations, the mean comparisons indicated $\alpha < 0$ for all calculated means. The negative value of growth associated coefficient (α) indicated that laccase is classified as non-growth related product. Similar observation was made by Tevatia *et al.* (2012). According to Luedeking and Piret (1959), all product formation during the non-growth activities is in accordance to the coefficients for non-growth associated product (β).

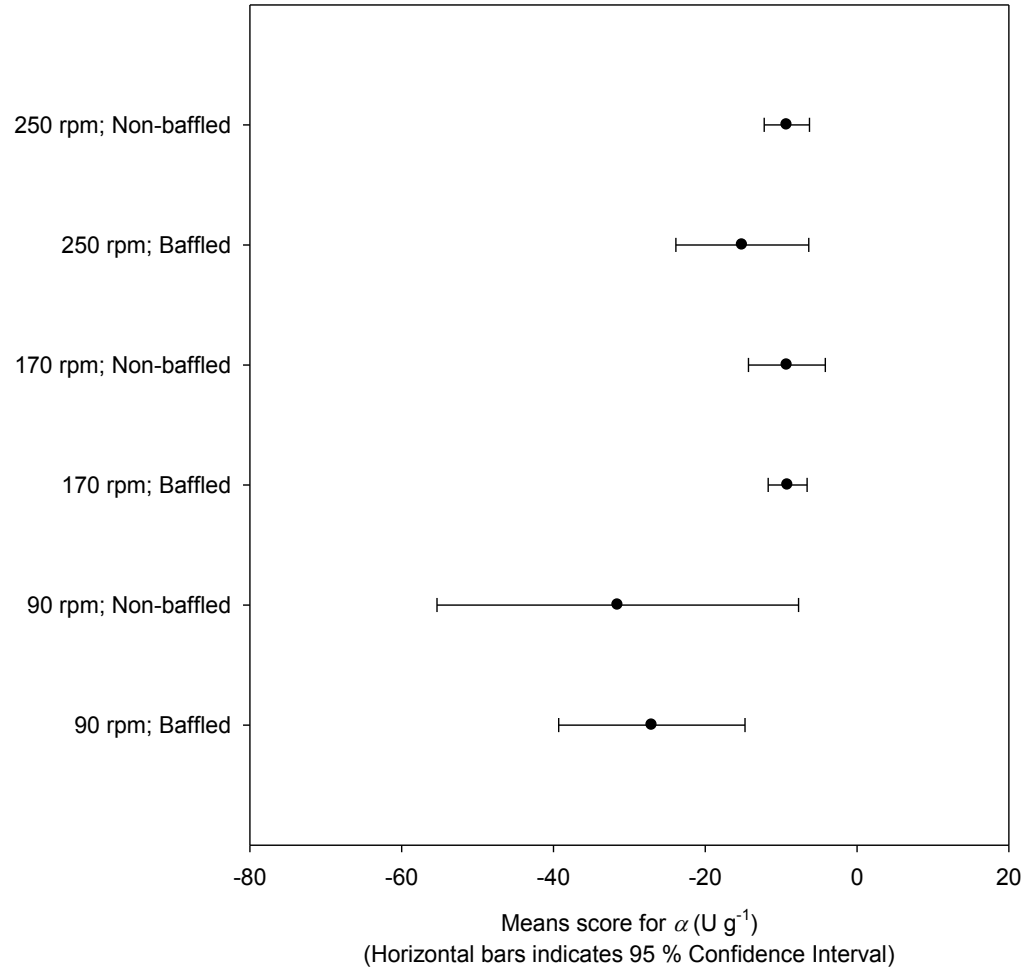


Figure 4.11: Multiple mean comparisons of α in baffled and non-baffled flask

The effect of baffled and non-baffled flasks on (β) value indicated insignificant differences at all agitations (Figure 4.12). Therefore, laccase production is independent from fungal growth activity since the effect of baffled and non-baffled only significantly influenced fungal growth activity at certain conditions.

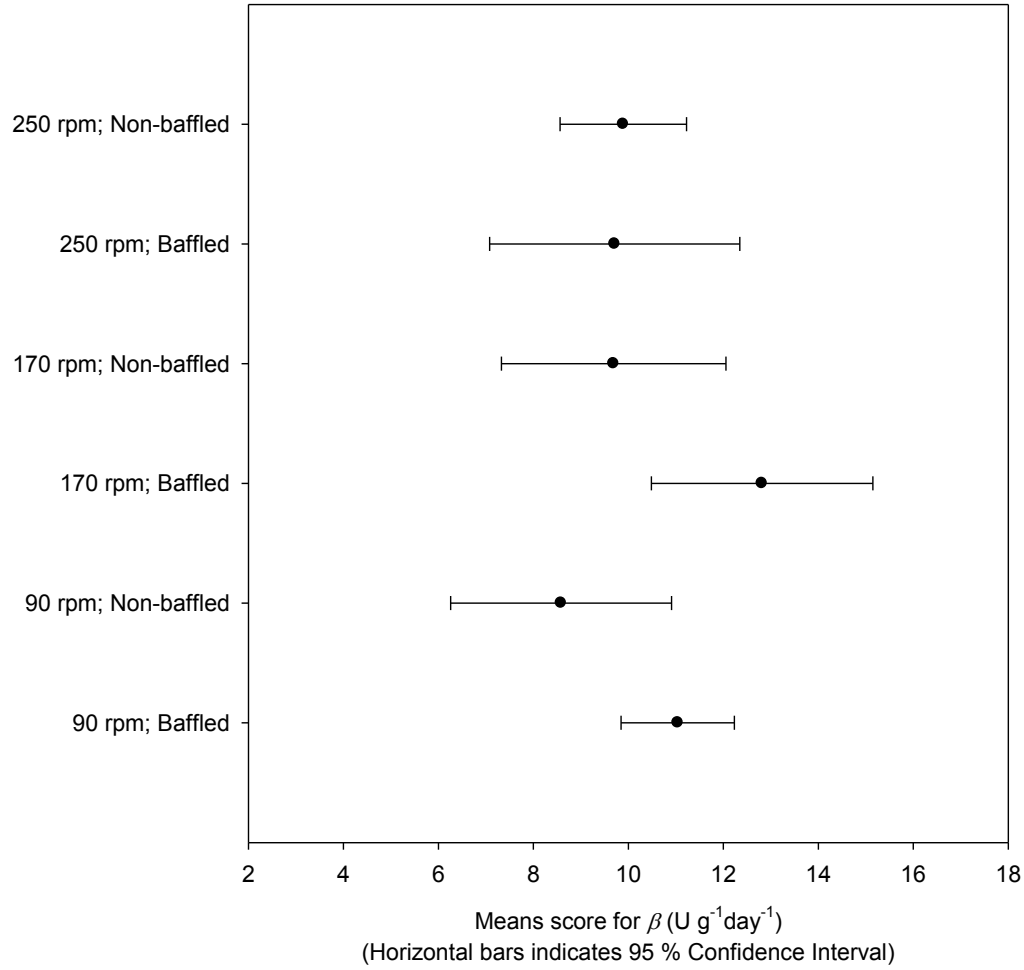


Figure 4.12: Multiple mean comparisons of β in baffled and non-baffled flasks

4.4 Simulation of Kinetic Model in Baffled and Non-baffled Flasks

Simulation of the kinetic models describing the rate of biomass formation (*dX/dt*), the rate of glucose utilization (*dS/dt*) and the rate of laccase production (*dP/dt*) were performed using Polymath 6.0. The differential equations were solved using the parameters shown in Table 4.7 and 4.8. The curve fitting for the cultivation in baffled and non-baffled flasks are shown in Figure 4.13 and 4.14, respectively. The simulations and the experimental data showed a good agreement. The coefficient correlation (R^2) for each kinetic model used in the baffled and non-baffled flasks is shown in Table 4.9.

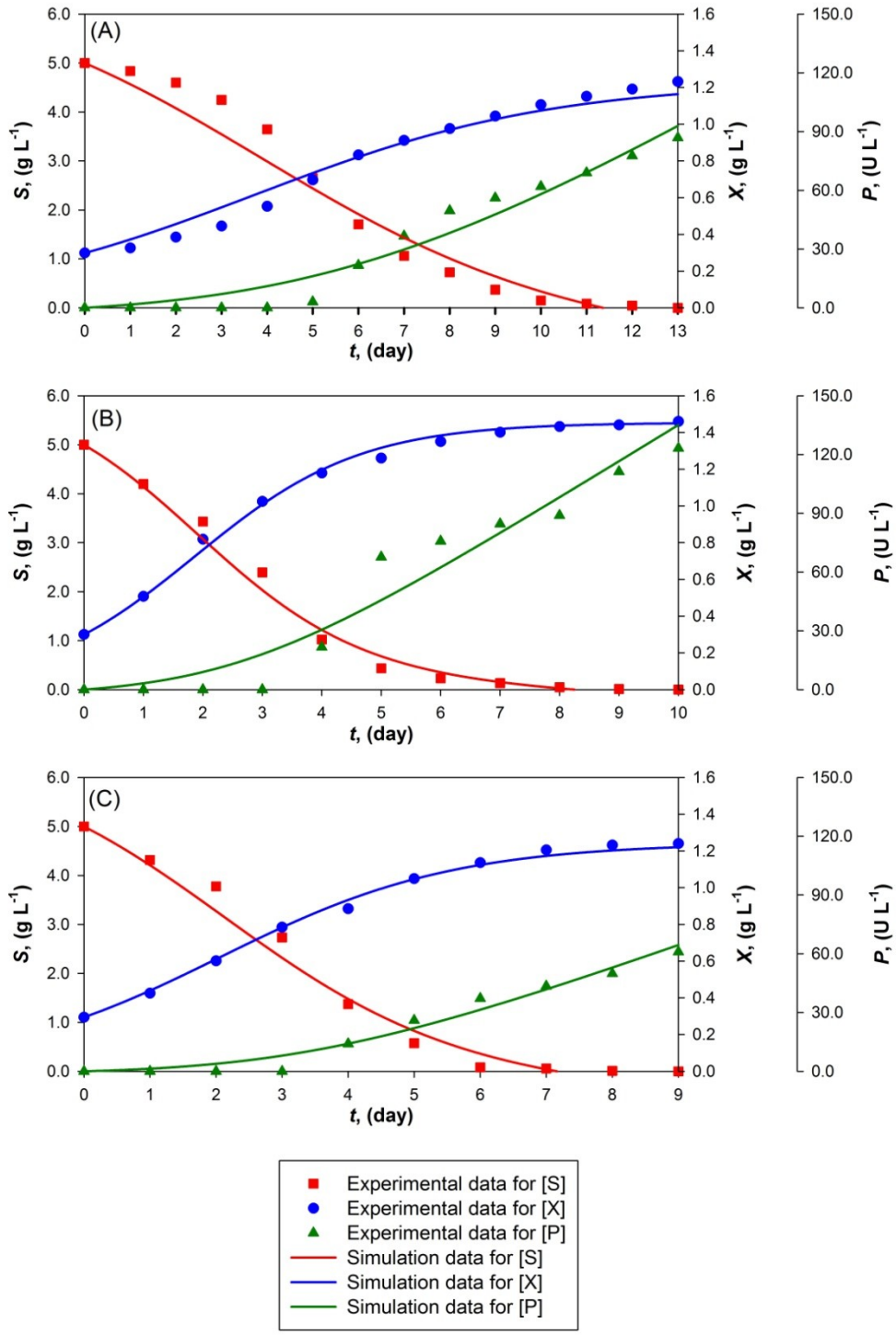


Figure 4.13: Kinetic model simulation for cultivation in baffled flasks: (A) 90 rpm, (B) 170 rpm and (C) 250 rpm

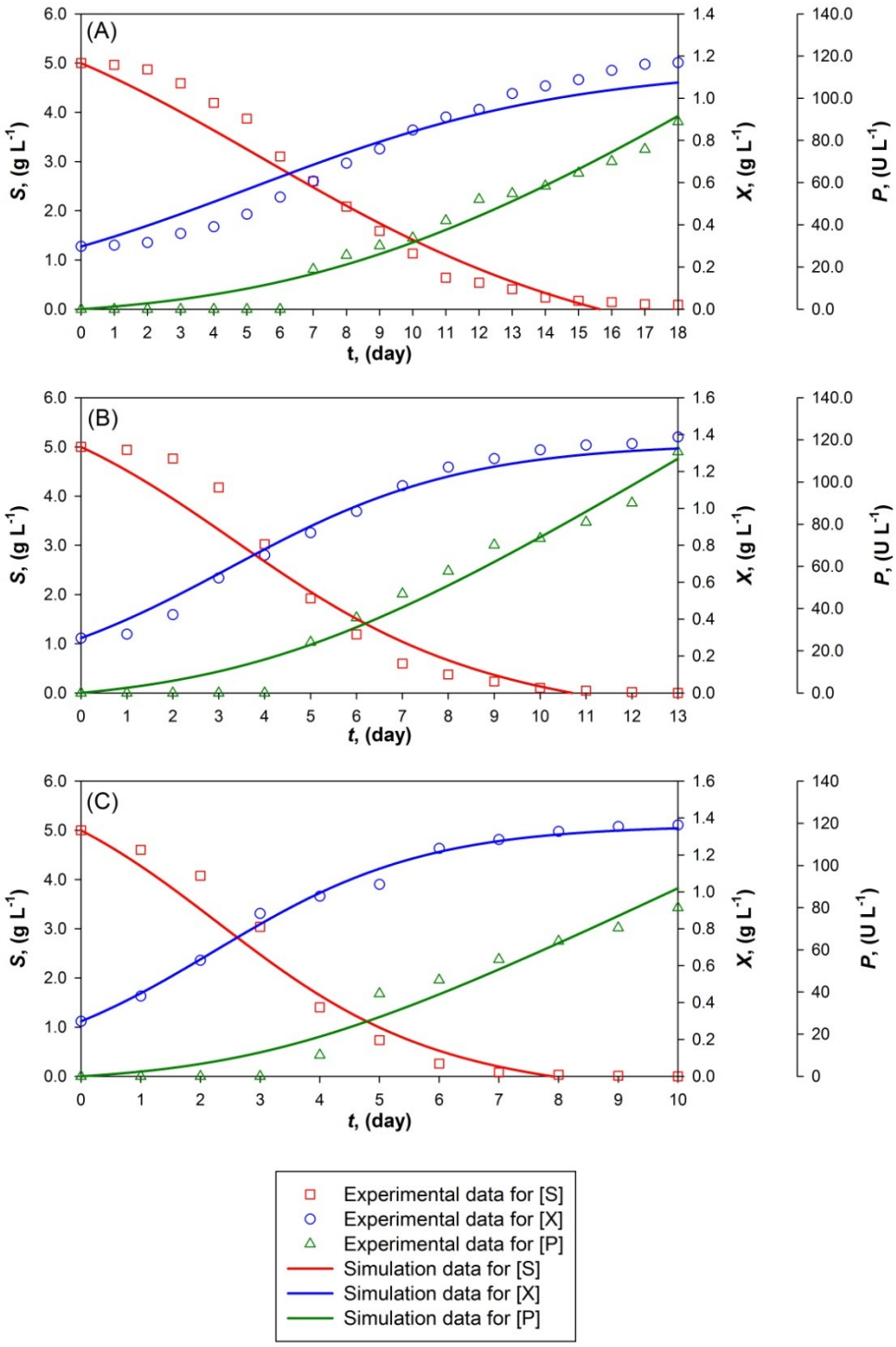


Figure 4.14: Kinetic model simulation for cultivation in non-baffled flasks: (A) 90 rpm, (B) 170 rpm and (C) 250 rpm

Table 4.9: Correlation coefficient (R^2) for the simulation of experimental data in shake flasks

Model Simulation	R^2		
Baffled flasks	**90 rpm**	**170 rpm**	**250 rpm**
Fungal growth $\frac{dX}{dt} = \mu_{max}(X)\left(1 - \frac{X}{X_{max}}\right)$	0.9949	0.9979	0.9983
Glucose utilization $-\frac{dS}{dt} = \frac{1}{Y_G}\left(\frac{dX}{dt}\right) + m_s(X)$	0.9897	0.9955	0.9932
Laccase production $\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta(X)$	0.9777	0.9703	0.9852
Non-baffled flasks	**90 rpm**	**170 rpm**	**250 rpm**
Fungal growth $\frac{dX}{dt} = \mu_{max}(X)\left(1 - \frac{X}{X_{max}}\right)$	0.9897	0.9972	0.9957
Glucose utilization $-\frac{dS}{dt} = \frac{1}{Y_G}\left(\frac{dX}{dt}\right) + m_s(X)$	0.9913	0.9876	0.9905
Laccase production $\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta(X)$	0.9842	0.9846	0.9741

Based on the profiles, the actively growing phase of fungal in baffled and non-baffled flasks coincided with rapid glucose utilization rate. The stationary phase for fungal growth is characterized by complete glucose utilization. The time for complete glucose consumption in baffled flasks was approximately observed on 11th, 8th and 6th day at agitations of 90, 170 and 250 rpm, respectively. As in the non-baffled flasks, the approximate time for complete glucose consumption was at 90, 170 and 250 rpm, which were observed on 17th, 11th and 8th day, respectively. According to Kelly *et al.* (2004), glucose utilization rate was significantly higher at high agitations due to the aggregation of cells which increased the availability of substrate for fungal pellet. This could probably explain the decreased of approximate time for complete glucose consumption by fungal cell as agitation was increased.

The start of laccase secretion was approximately observed on the 5th day for cultivation at 90 rpm with cultivations at 170 and 250 rpm indicated on the 4th day of the cultivations. Meanwhile in the non-baffled cultivations, the start of laccase secretion occurred on the 7th, 5th and 4th day for agitations at 90, 170 and 250 rpm, respectively. With respect to the glucose utilization, laccase production was observed during the period when glucose utilization rate is high. In addition, the highest rate of laccase production was apparently observed from the reduced growing phase towards the stationary phase in fungal growth. This observation corroborated with previous studies which stated that laccase production is influenced by rapid glucose consumption rate at the end of actively growing phase of the white rot fungi (Antecka *et al.*, 2009; Jiang *et al.*, 2009; Bettin *et al.*, 2011; Shi *et al.*, 2012).

Further increased in the profile of laccase production during the stationary growth phase suggested that laccase appeared not to be dependent upon growth rate. The profile also corroborated the estimated kinetic parameters of laccase production which $\alpha < \beta$ (Figure 4.11 and 4.12) as previously discussed in Section 4.3.3. Thus, it is obvious that laccase production is not associated with the cell metabolic pathways involved in the generation of ATP. It was previously reported that ligninolytic systems of white rot fungi are mainly activated during the secondary metabolic phase of the growth (Hammel, 1997). However, laccase production could also influence by the limiting substrate such as nitrogen source. According to Buswell and Eriksson (1994), the ligninolytic system of white rot fungus is triggered by nitrogen depletion.

4.5 Fermentation in Stirred Tank Reactor (STR)

4.5.1 Determination of Oxygen Volumetric Mass Transfer Coefficient (k_La) in STR

Liquid medium as indicated in Table 4.4 (Section 4.1.5) was used for the k_La studies in STR. The effect of aeration and agitation on the k_La for 2 Litre STR (Table 4.10) showed significant different between treatments ($p < 0.05$). From the study, the k_La value increased with agitation rate. At higher agitation, the flow behaviour was categorized as turbulent flow based on the calculated Reynolds number (Re_i) which was indicated in Table 4.10. The value of Re_i is dimensionless number which described the behaviour of fluid flow. For the STR, the Re_i values was calculated based on Equation 4.3 described by García-Ochoa and Gómez (1998):

$$Re_i = N_i \times D_i^2 \times \rho \times \mu^{-1} \qquad \text{Equation 4.3}$$

where N_i is the stirrer speed (s^{-1}), D_i is the impeller diameter (m), ρ is the fluid density (kg m^{-3}) and μ is the fluid viscosity (kg m^{-1} s^{-1}). Density and viscosity of normal distilled water at 25 °C was used in the calculation. As previously studied, the general range of k_La values normally found in STR is in the range of 0.05 – 0.3 s^{-1} (Mehta and Sharma, 1971). Based on Table 4.10, the turbulent flow is shown to be effective for mixing with significant increment in the k_La value. At higher Re_i value, laccase production increase significantly regardless of aeration rate at 600 rpm agitation.

Table 4.10: Effect of agitation on the k_La and Re_i for laccase production STR

Agitation (rpm)	Aeration (L min^{-1})	k_La (s^{-1})	Re_i	Laccase Production* (U L^{-1})
100	1.00	0.00418 ± 0.00040	4668	24.9 ± 1.6
	1.50	0.00544 ± 0.00055		25.7 ± 1.9
600	1.00	0.03138 ± 0.00204	28008	58.4 ± 1.0
	1.50	0.02694 ± 0.00196		59.6 ± 0.9

± Standard deviation
*Laccase production after 5 days of cultivation

Laccase production showed significant increased when k_La values increase at 600 rpm. At the same agitations, the effect of laccase production was found not significant when aeration increase although the k_La values showed significant different. This observation was correlated with further optimization of operating parameters for laccase production in STR.

4.5.2 Inoculum for Stirred Tank Reactor (STR) Cultivation

Determination of biomass for the inoculum in STR was done until biomass growth reach stationary phase. The biomass profile using GYMP growth medium is shown in Figure 4.15 A. Inoculum for STR cultivation was taken from the exponential phase. By using semi log plot, biomass concentration (X) at the fourth day (t = 4) was used for the STR inoculum (Figure 4.15 B).

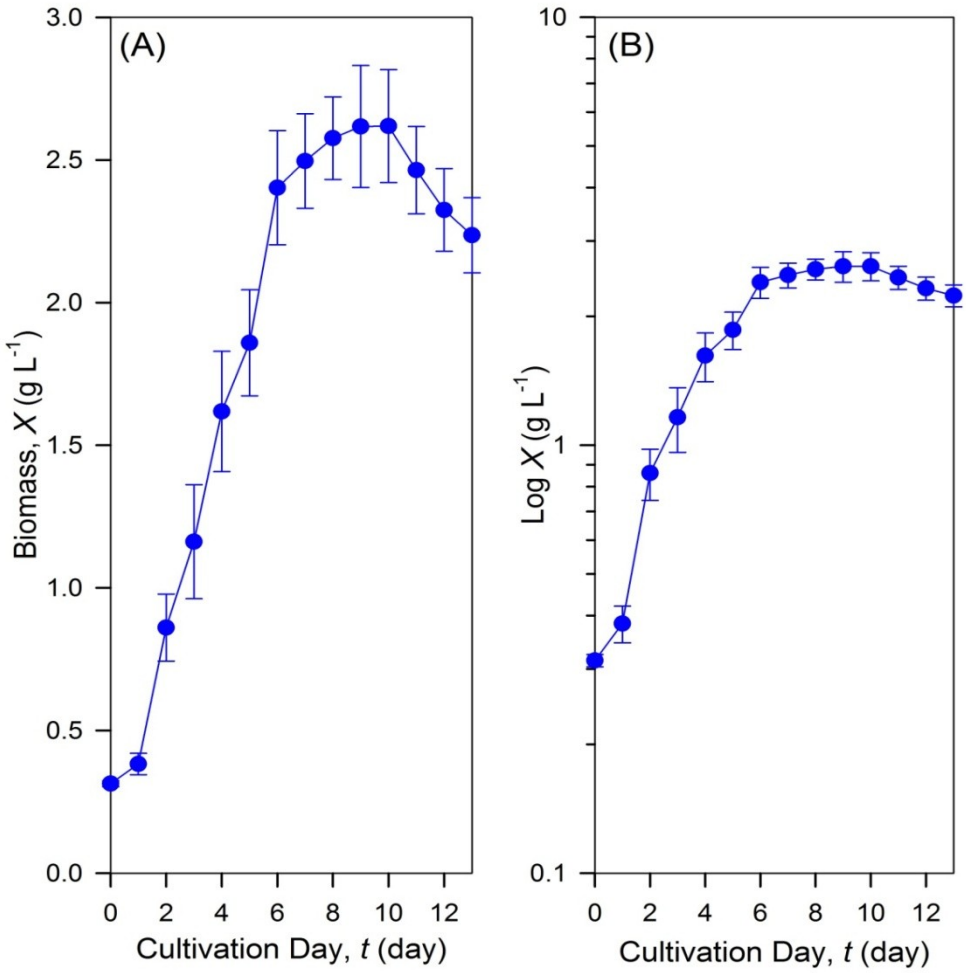


Figure 4.15: Growth profile using GYMP growth medium: (A) Biomass concentration profile and (B) Semi log plot of biomass profile (Error bar indicate standard deviation)

4.5.3 Screening of Selected Operating Variables of Stirred Tank Reactor (STR) using Full Factorial Design (FFD)

Laccase production in STR was carried out using 2 L Biostat B Plus (Sartorius, Germany) with automated control of temperature, pH and aeration rate. Unlike shake flasks, the use of STR for enzyme production enables the studies of parameters such as agitation, pH and aeration. The effect of selected operating variables namely agitation rate (rpm), aeration rate (L min^{-1}) and pH were screened using 2-level FFD. Fermentations were performed using liquid medium

described in Table 4.4 (Section 4.1.5) with 25 g L^{-1} of glucose concentration was used as carbon source. Response for operating variables screening including three Centre point experiments is shown in Table 4.11.

Table 4.11: Responses of FFD experiment on the screening of selected STR operating variables

Runs	Aeration (L min^{-1})	Agitation (rpm)	pH	Laccase Production (U L^{-1})
1	1.00	600	5	59.2
2	1.50	100	5	27.8
3	1.00	600	5	57.3
4	1.50	600	5	59.6
5*	1.25	350	4	49.1
6*	1.25	350	4	53.5
7	1.00	600	3	6.6
8	1.50	100	5	25.3
9	1.00	600	3	9.8
10	1.50	100	3	0.0
11	1.00	600	3	8.5
12	1.50	100	5	24.1
13*	1.25	350	4	50.4
14	1.50	100	3	0.0
15	1.50	600	3	6.4
16	1.50	600	5	58.7
17	1.00	100	3	0.0
18	1.00	100	5	26.7
19	1.00	100	5	23.8
20	1.50	100	3	0.0
21	1.00	600	5	58.7
22	1.00	100	5	24.3
23	1.00	100	3	0.0
24	1.50	600	5	60.5

*Centre point experiment

The highest laccase production was observed when aeration rate, agitation and pH were set at 1.50 L min^{-1}, 600 rpm and pH 5.0 respectively. Zero laccase production observed at agitation speed 100 rpm with respect to all conditions at pH 3.0. However at agitation speed 600 rpm, all combinations with pH 3.0 showed low laccase production.

4.5.4 Analysis of FFD Experiments on Screening of Selected Operating Variables

Factorial analysis using analysis of variance (ANOVA) using full order model terms (main-, 2-way and 3-way effects) was performed. The ANOVA analysis was generated by the Minitab 16 software (Table 4.12).

Table 4.12: ANOVA analysis for the effect of aeration, agitation and pH on laccase production in STR

Factor	DoF	Adj SS	Adj MS	*F*	*P*
Main factor	3	11,434.9	3,811.64	2,198.09	0.000
Aeration		0.0	0.03	0.02	0.891
Agitation		2,499.0	2,499.00	1,441.12	0.000
pH		8,935.9	8,935.90	5,153.12	0.000
2-way interactions	3	1,063.0	354.33	204.33	0.000
Aeration*Agitation		1.4	1.35	0.78	0.389
Aeration*pH		6.9	6.93	4.00	0.061
Agitation*pH		1,054.7	1,054.70	608.22	0.000
3-way interactions	1	2.7	2.73	1.58	0.225
Aeration*Agitation*pH		2.7	2.73	1.58	0.225
Centre Point	1	2,110.0	2,110.00	1,180.96	0.000
Error	18	31.2	1.73		
Total	26	14,641.8			

DoF: Degrees of freedom; Adj SS: Adjusted sum of squares; Adj MS: Adjusted mean of squares; *F*: F-statistic; *P*: P-statistic.

The full order term model showed good correlation with R^2 and R^2 *adjusted* value of 0.9979 and 0.9969 respectively. The effect of agitation, pH and their interaction (agitation*pH) were significant in laccase production using STR. The effect of centre points was significant in the analysis suggesting that the response was not linear with the level factor used. The effect of aeration, and the interactions of Aeration*Agitation and Aeration*pH were not significant in the experiment. The effect of full interaction (Aeration*Agitation*pH) was not significant as main effect aeration was not significant. The *F* value for the factors

studied showed pH (F = 5,153.12) has the strongest influence on the response as compared to the least pronounced factor which is Aeration (F = 0.02).

4.5.5 Residual Analysis of FFD Experiments on Screening of Selected Operating Variables

The main purpose of residual plot analysis is to validate the model which was used to fit the data. For an adequate model, the residuals should be structureless (Montgomery, 2001). Graphical analysis of residuals with standardized residual as comparison is shown in Figure 4.16. There was no unusual pattern for each plot. The was also no evidence of outliers as all standardized residual were well within the range of $-3 \leq d_i \leq +3$ (Figure 4.16 a). The variance of standardized residual and fitted value (Figure 4.16 b) did not show any increasing trend as fitted value increases. The analyzed data followed approximately normal distribution (Figure 4.16 c). The effect of the observation order did not influence the overall response as there was no strong observable pattern (Figure 4.16 d).

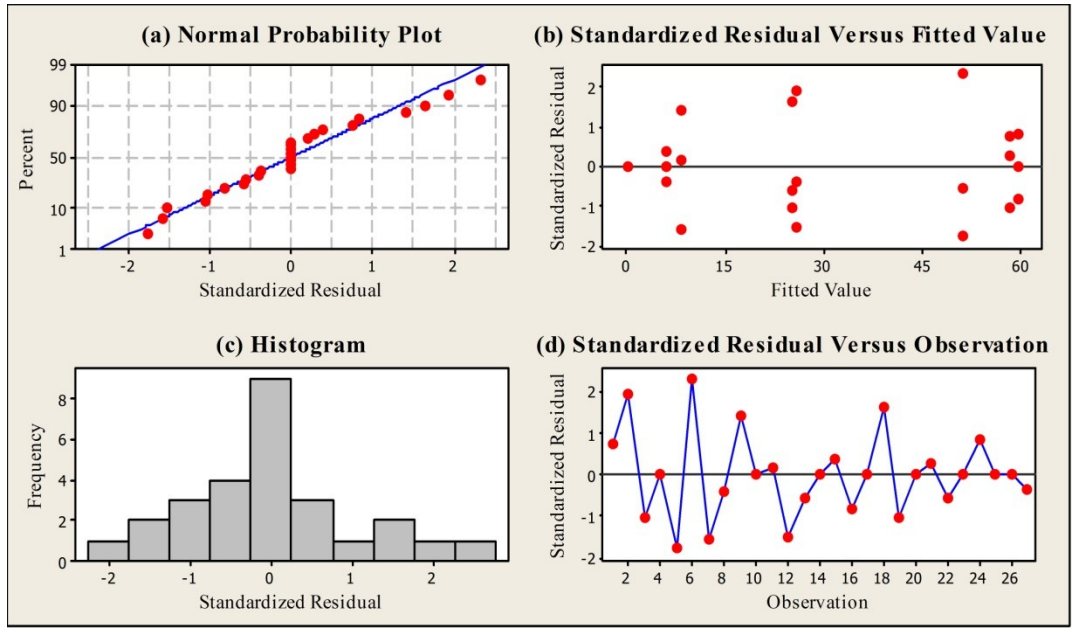


Figure 4.16: Residual plots of FFD model for the effect of aeration, agitation and pH on laccase production in STR

4.5.6 Main Effect Plot of FFD Experiments on Screening of Selected Operating Variables

The response for main effect plots was based on mean of observed laccase production for each factor level. The slope in main effect plot can be used to determine the magnitude of response change with respect to variable change (Figure 4.17). From the main effect plot, the slope of pH was the steepest as pH increased from 3 to 5 with the mean response (laccase activity) showed high increase. However the slope for agitation and aeration were not pronounced and mean response showed only minor changes with respect to variable change.

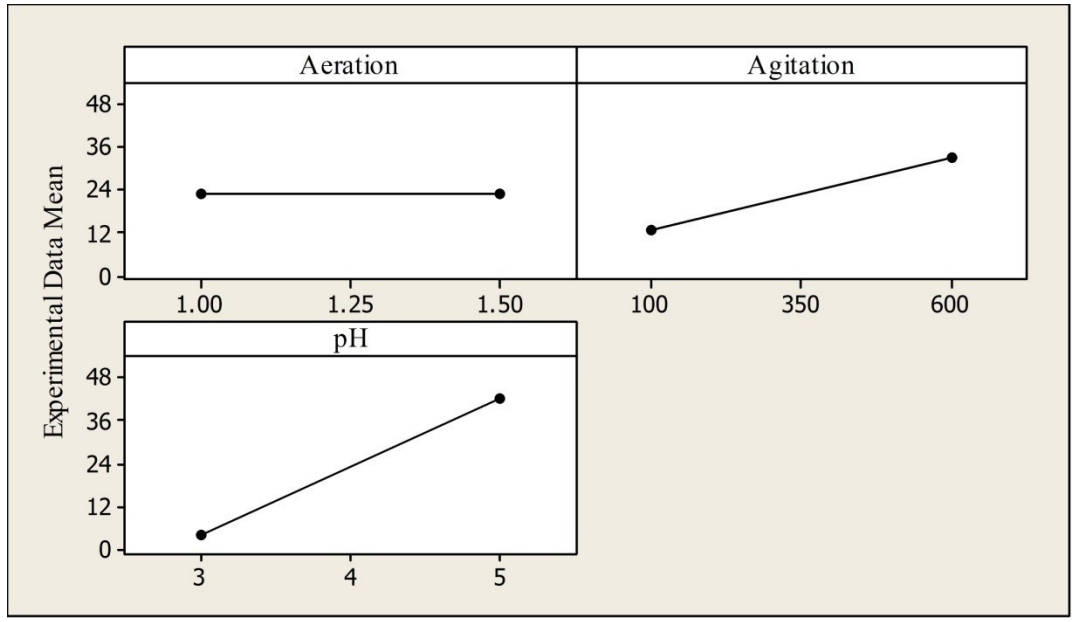


Figure 4.17: Main effect plot of FFD experiments on the effect of aeration, agitation and pH on laccase production in STR

4.5.7 Optimization of Selected Operating Variables of Stirred Tank Reactor (STR) using Face-Centred Central Composite Design (FCCCD)

Optimization of selected operating variables for laccase production in STR was performed using Face-Centred Central Composite Design (FCCCD). Based on the ANOVA analysis (Section 4.5.4) and the main effect plot (Section 4.5.6), the effects of pH and agitation were significant. Therefore these two parameters were considered as factors for the optimization process. However the effect of aeration rate was not significant. Thus the aeration rate was fixed at low level when performing the optimization experiment.

Face-Centred Design is a form of Central Composite Design (CCD) with α = 1 and is flexible in providing information on the response surface optimization experiment with less number of experimental runs. In fact, the FCCCD can be run sequentially as it has three types of point which are factorial points (cube points), axial points and Centre points. A FCCCD design is carried out by adding axial and Centre points into existing cube points of the factorial design.

In this study, the FCCCD experiment was performed in sequential by using the response from the FFD experiment. Minitab 16 software was used to design the FCCCD for the optimization of selected STR operating variables for laccase production (Table 4.13). The cube points used for the FCCCD were based on the factor setting in the FFD experiment. The axial and Centre points in FCCCD were located by the software.

Table 4.13: Responses of FCCCD experiments on the optimization of selected STR variables

Runs	Point type	Condition		Response	
		Agitation	pH	Experimental	Predicted
1	Axial	350	5.0	41.6	41.2
2	Axial	350	5.0	41.8	41.2
3	Axial	600	4.0	59.7	61.8
4	Centre	350	4.0	49.1	51.1
5	Centre	350	4.0	51.2	51.1
6	Cube	600	5.0	59.2	58.2
7	Axial	100	4.0	40.8	41.6
8	Centre	350	4.0	50.4	51.1
9	Centre	350	4.0	51.4	51.1
10	Cube	100	5.0	26.7	25.4
11	Centre	350	4.0	51.6	51.1
12	Axial	100	4.0	42.6	41.6
13	Axial	600	4.0	63.7	61.8
14	Centre	350	4.0	51.0	51.1
15	Cube	600	5.0	57.3	58.2
16	Centre	350	4.0	50.6	51.1
17	Cube	600	3.0	6.6	8.0
18	Cube	600	3.0	9.8	8.0
19	Cube	600	5.0	58.7	58.2
20	Centre	350	4.0	51.6	51.1
21	Centre	350	4.0	51.3	51.1
22	Centre	350	4.0	50.6	51.1
23	Axial	100	4.0	44.1	41.6
24	Cube	100	5.0	23.8	25.4
25	Cube	100	3.0	0.0	0.4
26	Centre	350	4.0	51.9	51.1
27	Axial	600	4.0	60.2	61.8
28	Centre	350	4.0	50.1	51.1
29	Centre	350	4.0	51.1	51.1
30	Cube	100	3.0	0.0	0.4
31	Axial	350	3.0	2.8	3.6
32	Axial	350	3.0	4.9	3.6
33	Cube	100	5.0	24.3	25.4
34	Centre	350	4.0	51.6	51.1
35	Cube	600	3.0	8.5	8.0
36	Axial	350	5.0	40.8	41.2
37	Centre	350	4.0	51.3	51.1
38	Cube	100	3.0	0.0	0.4
39	Axial	350	3.0	3.4	3.6

4.5.8 Analysis of FCCCD Experiments on Optimization of Selected Operating Variables

FCCCD analysis using ANOVA was carried out using Minitab 16 with linear and quadratic model used to fit the experimental data. The level of significance was set at α = 0.05 with P value less than α value indicating significant effect. The ANOVA analysis on FCCCD linear model is shown in Table 4.14.

Table 4.14: ANOVA and descriptive statistics for the FCCCD linear model

Descriptive Statistic					
R^2	0.4946				
R^2 adjusted	0.4665				
S_{Error}	15.2412				
PRESS	10,091.4000				
ANOVA					
Term	**DoF**	**Adj. SS**	**Adj. MS**	***F***	***P***
Linear	2	8182.51	4091.26	17.61	0.000
Agitation		1828.11	1828.11	7.87	0.008
pH		6354.40	6354.40	27.35	0.000
Lack of fit	6	8325.44	1387.57	1120.10	0.000
Residual error	30	37.16	1.24		
Total	38	16545.11			

R^2 = Correlation coefficient; S_{Error} = Standard error regression; PRESS = Prediction sum of squares; DoF = Degrees of freedom; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean of squares; F: F-statistic; P: P-statistic

The linear model of FCCCD analysis showed the effect of agitation and pH were significant in the linear model. Since the lack of fit test indicated significant P value, the null hypothesis that the experimental data fitted well the linear model was rejected. In order to satisfy the fitting process, a higher polynomial model with quadratic terms was used (Table 4.15).

Table 4.15: ANOVA and descriptive statistics for the FCCCD quadratic model

Descriptive Statistic					
R^2	0.9974				
R^2 adjusted	0.9970				
S	1.1347				
PRESS	64.1949				
ANOVA					
Term	**DoF**	**Adj. SS**	**Adj. MS**	***F***	***P***
Linear	2	8182.5	4091.26	3177.32	0.000
Agitation		1828.1	1828.11	1419.73	0.000
pH		6354.4	6354.40	4934.91	0.000
Square	2	7845.1	3922.55	3046.30	0.000
Agitation*Agitation		3.2	3.18	2.47	0.126
pH*pH		6815.6	6815.57	5293.06	0.000
Interaction	1	475.0	475.02	368.91	0.000
Agitation*pH		475.0	475.02	368.91	0.000
Lack of fit	3	5.3	1.78	1.43	0.252
Residual error	30	37.2	1.24		
Total	38	16545.1			

R^2 = Correlation coefficient; S_{Error} = Standard error regression; PRESS = Prediction sum of squares; DoF = Degrees of freedom; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean of squares; *F*: F-statistic; *P*: P-statistic

The ANOVA analysis on quadratic model indicated the lack of fit (P = 0.252) was not significant. Thus, the null hypotheses of the quadratic model fitted well the experimental data is not rejected. The model also indicated P value for the square and the interaction terms were significant ($P < 0.05$) which refers to the curvature effect in the model. The detected curvature effect showed the possible optimum range where maximum laccase production could be predicted by the model. Optimum condition is determined within the range of curvature for maximum enzyme production (Saat *et al.*, 2012). Based on the descriptive statistic, the quadratic model has better correlation than the linear model. The low R^2 value in linear model suggested that model reduction from the higher polynomial is unlikely to result in a simpler model. Therefore a quadratic model is used in the FCCCD modelling on the selected STR operating variables for laccase production.

4.5.9 Determination of Equation for Optimized Condition

The general equation for the optimized condition is based on the estimated regression coefficient in Table 4.16. Regression coefficient was estimated in coded unit using Minitab 16 software.

Table 4.16: Estimated regression coefficient of quadratic model in coded unit

Term	Estimated Coef.	SE Coef.	*T*	*P*
Constant	51.0563	0.2720	187.684	0.000
Agitation	10.0778	0.2675	37.679	0.000
pH	18.7889	0.2675	70.249	0.000
Agitation*Agitation	0.6195	0.3942	1.572	0.126
pH*pH	-28.6805	0.3942	-72.753	0.000
Agitation*pH	6.2917	0.3276	19.207	0.000

Coef.: Coefficient; SE: Standard error; *T*: *t* statistic; *P*: *P*-Statistic.

The square term of Agitation*Agitation was not used in the equation as the *P* value for the term was not significant. The final equation describing the quadratic model for the optimized condition can be written in terms of coded unit as follows:

$$\text{Laccase (U L}^{-1}) = 51.0563 + 10.0778(A) + 18.7889\ (B) - 28.6805\ (BB) + 6.2917\ (AB) \quad \text{(Equation 4.4)}$$

where *A* is the variable for agitation, *B* is the variable for pH, *BB* is the variable for square of pH and *AB* is the variable for interaction between agitation and pH.

4.5.10 Response Surface Plot and Contour Plot on Optimization of Selected Operating Variables

The relationship of agitation and pH effects on response is shown in the 3-D surface plot generated from Minitab 16 software (Figure 4.18). The region in

surface plot where estimated optimum lies is denoted by the light green peak. Surface with dark green colour showed low laccase production. The lowest laccase production observed at the lower pH and agitation. At pH 3, increase in agitation did not result in significant large improvement on laccase production. In contrast, at pH 4 and 5, laccase production was markedly increased at different agitation rates.

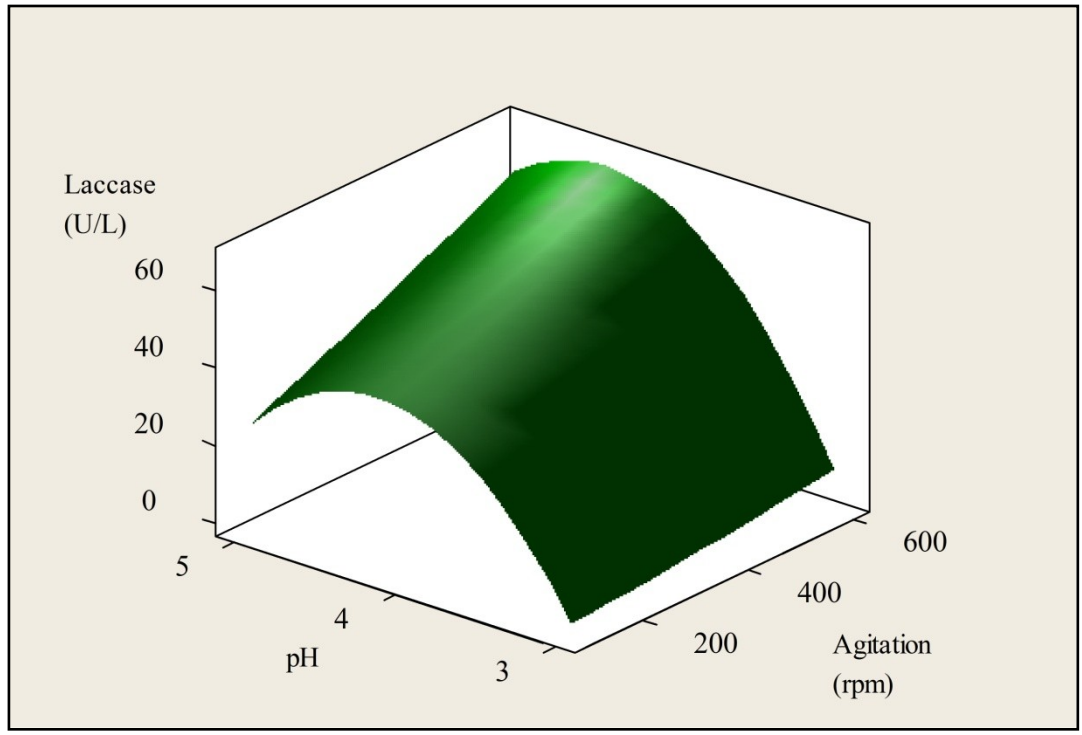


Figure 4.18: Response surface plot of agitation and pH effects on laccase production in STR

Figure 4.19 showed the contour plot of agitation and pH on laccase production generated using Minitab 16 software.

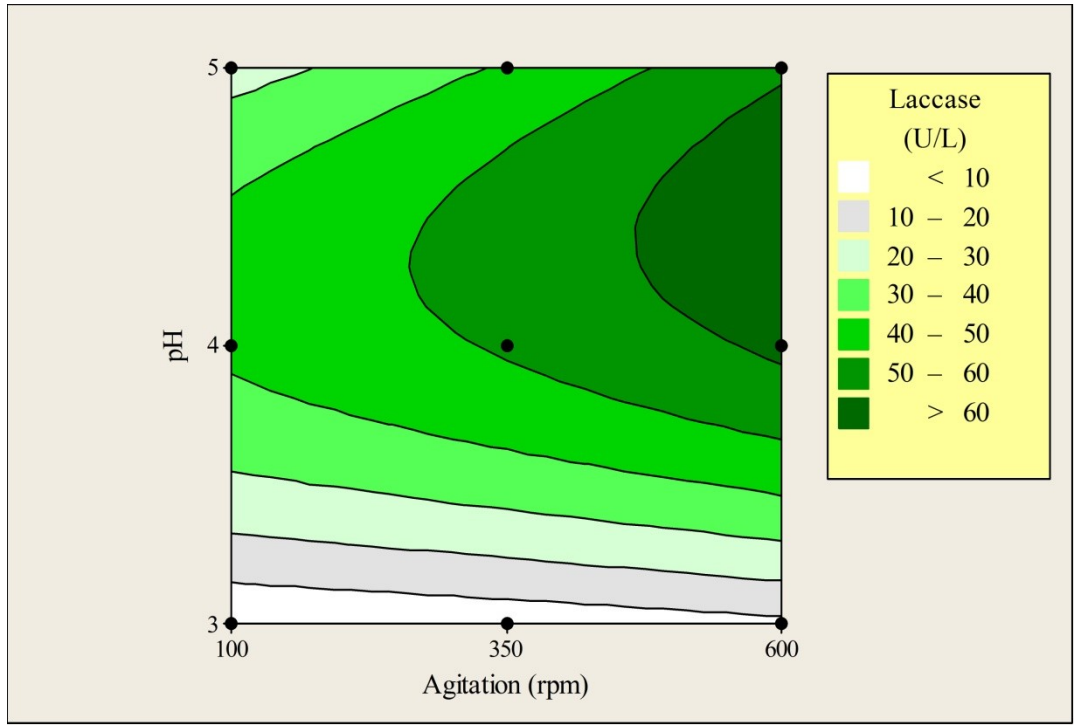


Figure 4.19: Contour plot of agitation and pH effects on laccase production in STR

The contour plot describes the response in the form of contour line. Unlike the 3-D plot, the contour plot is represented with factors plotted on *x* and *y*-linear scales. The response values are represented as contour. From the contour plot, maximum productivity with laccase yield ≥ 50 U L^{-1} was observed above pH 4.0 with agitation speed 350 rpm. Low laccase production (≤ 10 U L^{-1}) would be observed under pH 3.0 at all agitation rate settings.

4.5.11 Residual Plot Analysis of FCCCD Experiments on Optimization of Selected Operating Variables

The residual analysis was performed in order to evaluate the fitted model. Standardized residual was used in the analysis. All residual plots were generated as part of the ANOVA analysis using Minitab 16 software. The normal probability plot (Figure 4.20 a) showed all standardized residuals fell on the straight line. A

random scatter pattern observed (Figure 4.20 b) with standardized residual at negative and positive side are well located within acceptable ± 2 residual range as fitted value increases. The histogram (Figure 4.20 c) showed a typical normal distribution. Based on Anderson-Darling (*AD*) statistical analysis, the *AD* value is 0.173 (P = 0.922). The smaller Anderson-Darling value, the better the data fits to the normal distribution (Annuar *et al.*, 2010). Finally the effect of observation order (Figure 4.20 d) did not show any specific influence on the standardized residual as all data points were randomly scattered.

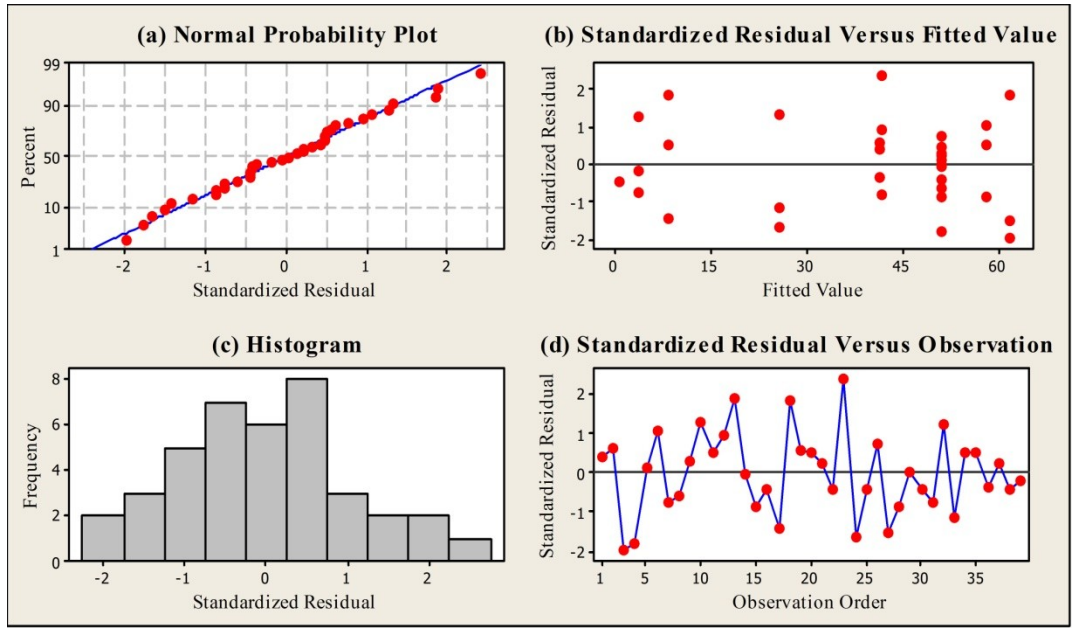


Figure 4.20: Residual plots of FCCCD experiments on the effect of agitation and pH in STR laccase production

4.5.12 Determination of Optimum Conditions and Validation Experiments

The determination of optimum condition was carried out using response optimizer application in the Minitab 16 software. The objective for the optimization was to achieve maximum laccase production with the predicted maximum laccase production calculated at 67.2 U L^{-1} with composite desirability,

D > 0.9. The validation experiments using the optimized condition for maximum laccase production is shown in Table 4.4. From the validation experiments data, it is clear that optimum conditions for laccase production by *P. sanguineus* in STR were correctly determined. Within the range of uncertainty determined (± 4.9), the actual laccase production of 62.9 U L^{-1} was closely matched the predicted laccase yield of 67.2 U L^{-1} (Table 4.17). The calculated composite desirability value suggested that the overall setting is well satisfied with the predicted response for maximum laccase production.

Table 4.17: Validation experiments under optimized conditions with maximum laccase production as the objective of the optimization

Validation Experiment Setting.	**Response (U L^{-1})**	
	Experiment	**Average**
Agitation 600 rpm; pH 4.43 and aeration 1.0 L min^{-1}.	67.0 58.6 62.5 68.6 57.6	62.9 ± 4.9

± Standard deviation

4.6 Fermentation Profiles in Stirred Tank Reactor (STR)

Experiments were carried out in 2 L STR according to the optimized conditions (Table 4.17) as mentioned in Section 4.5.12. Fermentations were performed using liquid medium described in Table 4.4 (Section 4.1.5) with 25 g L^{-1} of glucose concentration was used as carbon source. Fungal laccase production and glucose concentration in the medium was determined for every day until seventeen days of cultivation period. Meanwhile fungal biomass dry weight determination was performed at selected interval time as direct sampling from the culture medium was impossible due to the heterogeneous culture. Batch

fermentation profiles including biomass concentration (*X*), laccase production (*P*), and glucose concentration (*S*) in the STR is shown in Figure 4.21.

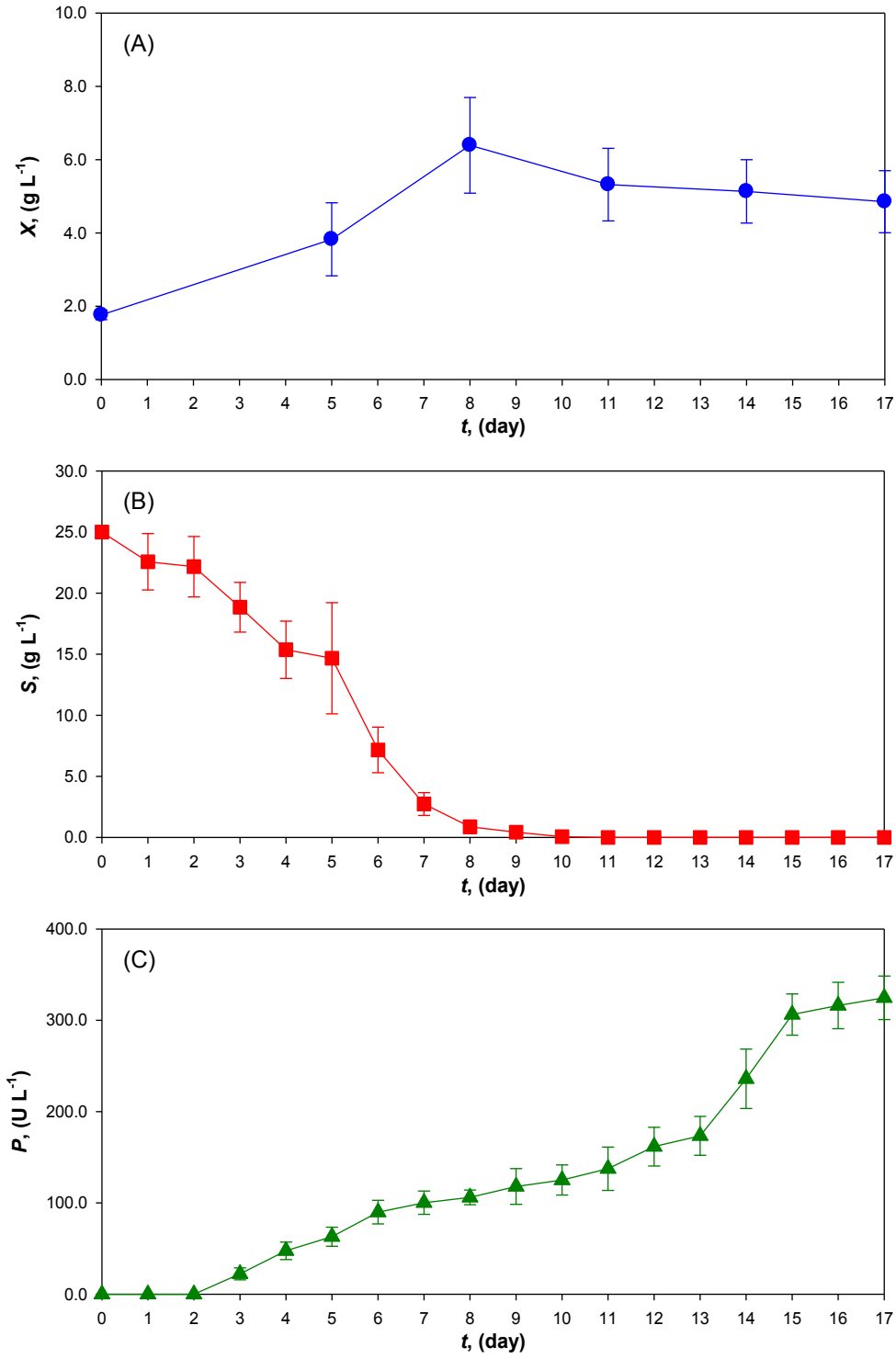


Figure 4.21: Batch fermentation profiles in STR: (A) Fungal growth, (B) Glucose utilization and (C) Laccase production (Error bar indicates standard deviation of 3 replicate experiments)

To determine the biomass from the STR culture, the whole culture was harvested at specific time. Thus, some variations observed for the biomass profile in STR cultivation were probably due to the samples from different batches. Nevertheless, the biomass profile obtained follows the general microbial growth trend. The biomass data at selected interval time was corroborated by the glucose concentration profile in the way that increasing biomass was followed by rapid utilization of glucose and maximum biomass production was observed as glucose was completely consumed. Based on the profile, biomass concentration was decreased from the 11th day until end of the cultivation period which was due to the depletion of carbon source (glucose) in the medium.

Based on the profile, the start of laccase production was observed after three days of cultivation. The production rate of laccase increased steadily up until the 13th day and increased rapidly thereafter. Laccase production in this study peaked up significantly when biomass reached the stationary growth phase. This observation was accompanied by the depletion of glucose on the 10th day of glucose concentration profile. In this case, fungal laccase was not actively produced during the highest rate of glucose utilization in the growth phase of the fungal population. As previously reported, laccase production is higher when glucose is limited in the culture medium (Tavares *et al.*, 2005; Thiruchelvam and Ramsay, 2007; Antecka *et al.*, 2009). On the other hand, laccase production is strongly independent from fungal growth due to the fact that biomass increase during the growth phase did not concomitantly caused increased in laccase production.

4.7 Kinetic Parameters Estimation and Simulations in Stirred Tank Reactor (STR)

All parameters for kinetic models of fungal growth (*dX/dt*), laccase production (*dP/dt*) and glucose utilization (*dS/dt*) were determined using non linear regression method with Polymath 6.0 software was used to calculate the regression analysis (Table 4.18). Simulations were performed by solving the differential equations using Polymath 6.0 software (Figure 4.22). The simulation of experimental data in STR cultivation showed good coefficient of correlation (R^2) for fungal growth, laccase production and glucose utilization (Table 4.19).

Table 4.18: Kinetic parameters of fungal growth, glucose utilization and laccase production in STR

Parameter	Value
μ_{max}	0.515 ± 0.270
X_{max}	5.406 ± 2.043
X_0	1.562 ± 0.827
Y_G	0.202 ± 0.100
m_S	0.098 ± 0.025
α	-7.050 ± 3.831
β	4.372 ± 0.879

(± 95% confidence interval)

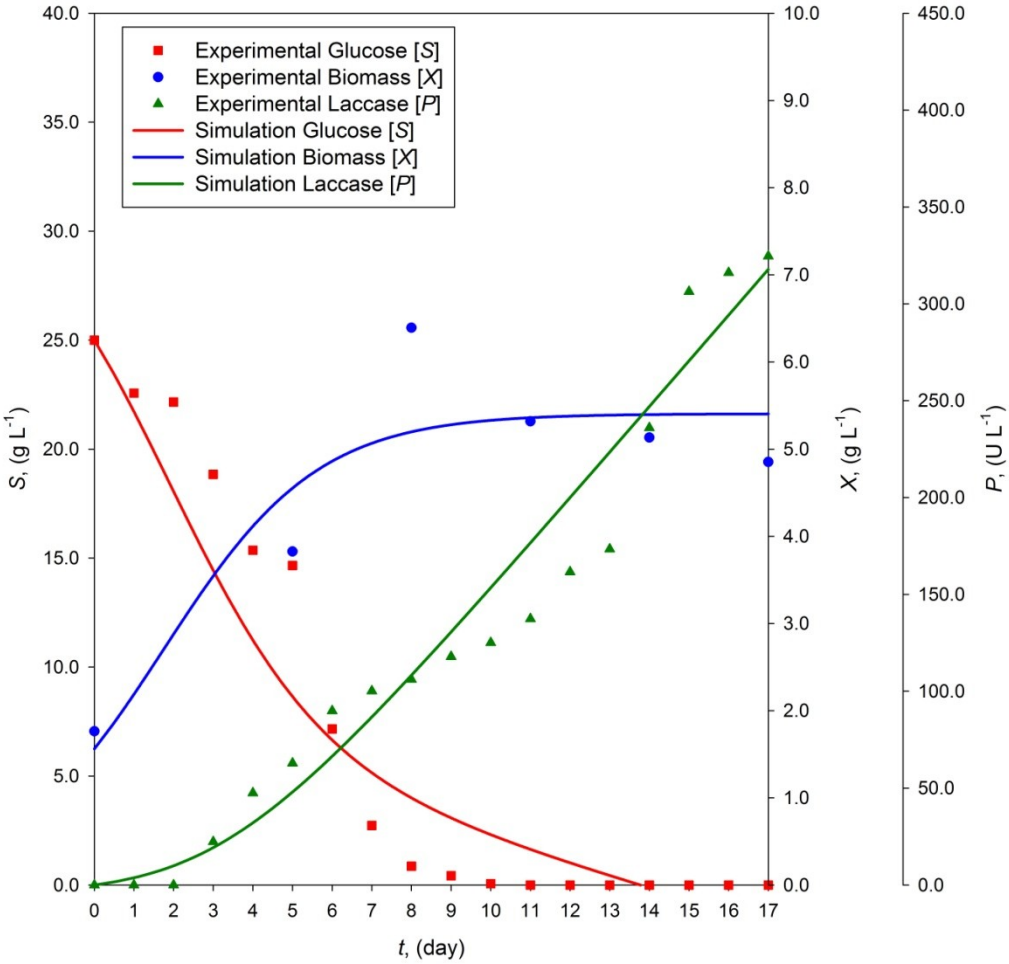


Figure 4.22: Kinetic model simulation in STR cultivation at optimum conditions (600 rpm, pH 4.43 and 1.0 L min^{-1})

Table 4.19: Correlation coefficient (R^2) for the simulation of experimental data in the STR

Model Simulation	Correlation coefficient, (R^2)
Fungal growth $\frac{dX}{dt} = \mu_{max}(X)\left(1 - \frac{X}{X_{max}}\right)$	0.9033
Glucose utilization $-\frac{dS}{dt} = \frac{1}{Y_G}\left(\frac{dX}{dt}\right) + m_S(X)$	0.9697
Laccase production $\frac{dP}{dt} = \alpha\left(\frac{dX}{dt}\right) + \beta(X)$	0.9755

The simulation of fungal growth showed slight differences between simulated and experimental biomass concentration (X) was probably due to the sampling method where slight variations from different batches were encountered. The profiles in STR cultivation indicated similar trend with the profiles obtained from the shake flasks cultivations. Thus, the kinetic model describing shake flasks fermentation could adequately fitted the experimental data in STR with controlled conditions e.g. pH and dissolved oxygen content.

In this study, the substrate dependent kinetic model such as Monod equation was not used for the growth kinetic study due to the lack of information on the segregated biomass concentration in STR cultivation. However, biomass production in STR was satisfactory modelled using the logistic growth model. Despite the difficulties in obtaining the biomass sample, the growth kinetic parameters showed only a small value of 95% confidence interval.

Multiple mean comparisons of the estimated growth kinetic parameters in STR and shake flask cultivations were performed and shown in Figure 4.23. The cultivation in baffled flasks at 170 rpm was used as comparisons since the growth kinetic parameters obtained at this agitation rate were the highest in all shake flasks cultivations. At controlled condition such as dissolved oxygen and pH in the medium, the maximum specific growth rate (μ_{max}) obtained from the STR cultivation showed insignificant difference compared to the shake flasks cultivation. However, maximum biomass concentration (X_{max}) in STR cultivations was significantly higher than the shake flasks cultivation. The higher X_{max} achieved in STR cultivation was due to higher inoculum source and initial glucose concentrations compared to the shake flasks cultivation.

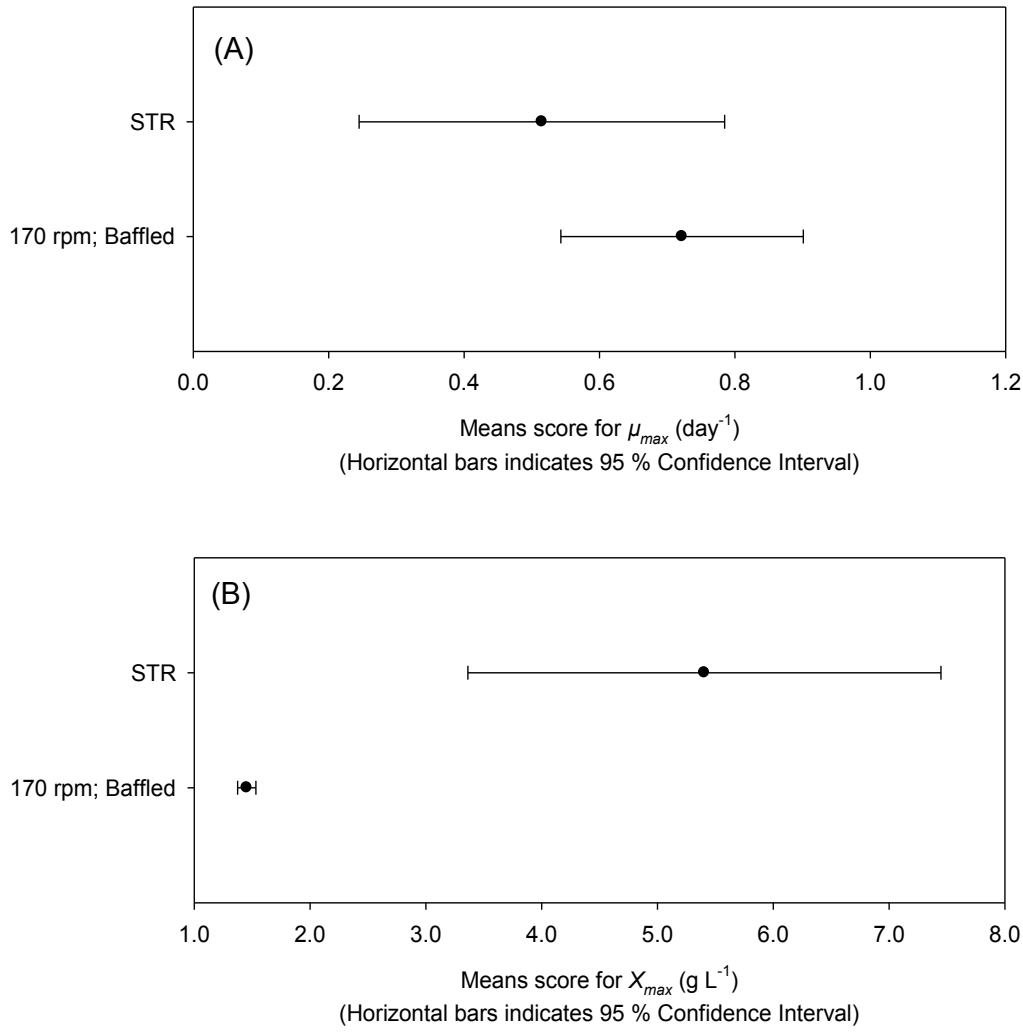


Figure 4.23: Multiple mean comparisons of growth kinetic parameters in STR and shake flasks; (A) μ_{max} and (B) X_{max}

Under controlled conditions such as pH and dissolved oxygen content, fungal growth rate in STR was not significantly different compared to the shake flasks cultivation. Based on the comparison, the insignificant difference of μ_{max} in both cultivation systems suggested fungal growth rate was not strongly influenced by the different cultivation conditions encountered inside the STR and shake flasks.

Increased in shear stress in the STR by high agitation rates lead to high energy dissipation which may result in fragmentation and damage to the pellets and mycelium. As indicated by (Kelly *et al.*, 2004), better cell segregation can be achieved when higher agitation was set at the early stage of the fermentations resulted in decreased of pellet concentrations so that each pellets has more substrate available. More substrate supply leads to bigger pellets formation and extended growth period for fungal population has been reported as the reason for increasing growth rate at higher energy dissipation (Cui *et al.*, 1998). However in our system, this was not observed.

Kinetics of laccase production was adequately modelled by using Luedeking-Piret model which was previously used in the kinetic modelling of lactic acid production (Venkatesh *et al.*, 1993; Amrane, 2001). Tavares *et al.* (2006) has studied the kinetic of laccase production from *Trametes versicolor* in shake flasks using Luedeking Piret model. The negative α value indicated that laccase production is independent on fungal growth. Since $\alpha < \beta$, laccase is mostly produced when biomass reach stationary phase ($dX/dt = 0$). Based on the simulation and experimental data, laccase production is strongly produced mainly in the stationary phase of the fungal growth. The start of laccase production was observed on the 3rd day of the cultivation period and steadily increased until end of cultivation period on the 17th day. The period of laccase production also coincided with rapid depletion of glucose which was approximately observed from the 2nd day until the 10th day when glucose concentration became completely depleted. The maximum production of laccase in this study was not available experimentally as the laccase profile in STR cultivation could still showed increasing trend even when the cultivation has been terminated on the 17th day.

A mean comparison between STR and shake flasks cultivations was also performed for the kinetic parameters of glucose utilization (Figure 4.24). As discussed earlier, baffled flasks at 170 rpm cultivations was used as comparison. The use of higher initial glucose concentration in the STR cultivation insignificantly increased the yield coefficient of biomass over substrate (Y_G). In contrast, the coefficient for cell maintenance (m_S) showed significant increased in the STR cultivations. In this case, more glucose has been channelled for cell maintenance rather than growth activity. The cultivation in STR can be associated with higher shear conditions, which may resulted in low Y_G coefficient due to fungal growth in the STR experiencing stress. This could explain why the m_S coefficient in STR increased by 2.4-fold compared to shake flasks.

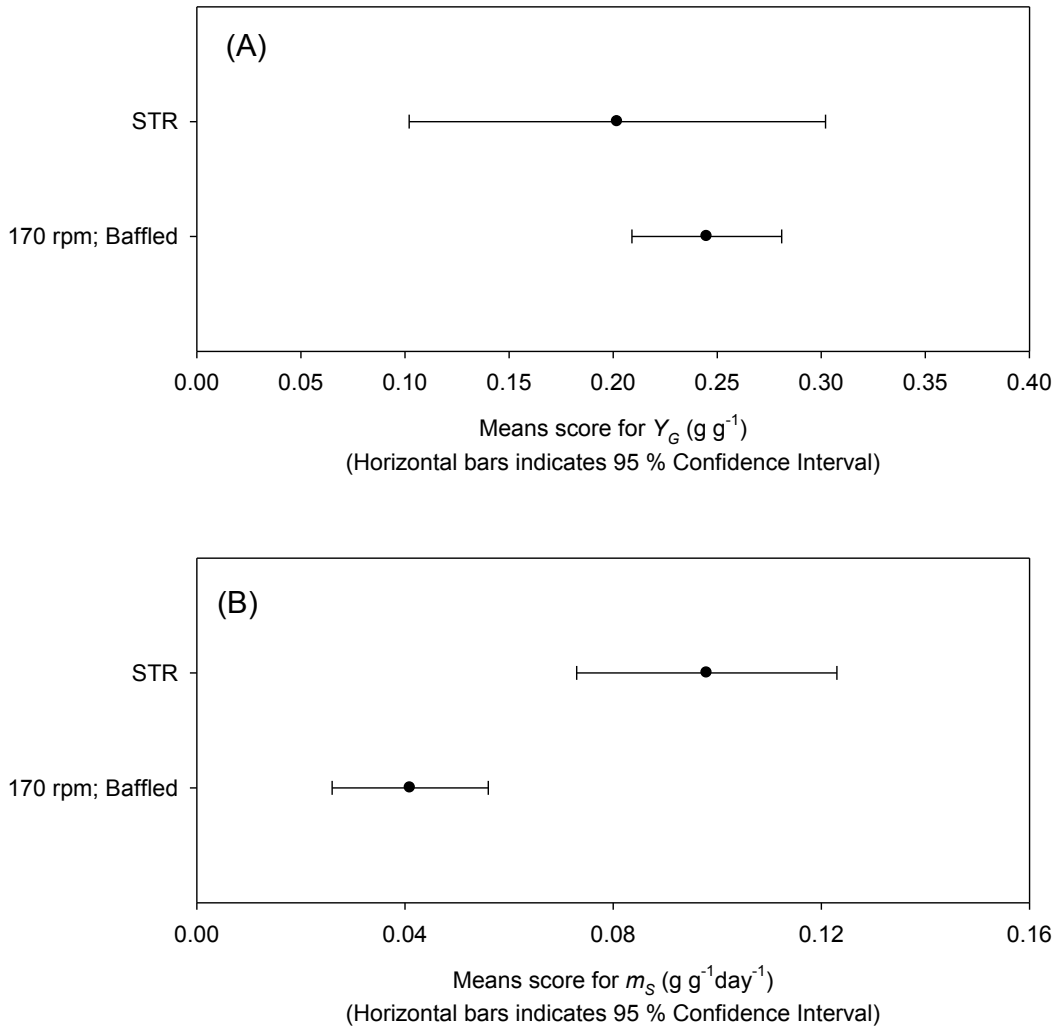


Figure 4.24: Multiple mean comparisons of glucose utilization kinetic parameters in STR and shake flasks; (A) Y_G and (B) m_S

4.8 Partial Purification of Crude Laccase Fermentation

Crude samples from the cultivation in stirred tank reactor (STR) and shake flasks cultivation were used for the partial purification. In the study, the steps used for the partial purification of laccase crude sample included ammonium sulfate precipitation and size exclusion chromatography using Sephadex as gel matrix in self packed column. First, the crude sample was precipitated using ammonium sulfate in order to concentrate protein from the liquid sample.

In the next step, protein precipitate was redissolved and loaded onto the desalting column with Sephadex G-25 as gel matrix. The impurities including protein with low molecular weight and salts were separated from the protein interest. During the desalting step, high molecular weight substances have the partition coefficient value of $K_{AV} = 0$ while the low molecular weight substances have K_{AV} value close to 1. The value of K_{AV} is a coefficient value in the selectivity curve of protein on different type of Sephadex (Grover and Kapoor, 1973).

The final step in the purification study involved the protein fractionation using Sephadex G-75. The gel matrix for the protein fractionation has to be selected properly so that protein sample do not elute with either void volume or total column volume. In the fractionation, the value of K_{AV} should be in the range $0 \leq K_{AV} \leq 1$ which located on the slope region of the protein selectivity curve on different matrix of Sephadex (Grover and Kapoor, 1973). Thus samples applied for fractionation are eluted closer and may overlap among the eluents.

4.8.1 Ammonium Sulfate Precipitation

Samples from the stirred tank reactor (STR) were used in the fractionation using ammonium sulfate precipitation. Laccase activity and protein concentration for each fraction of ammonium sulfate saturation are summarized in Table 4.20.

Table 4.20: Protein fractionation using ammonium sulfate precipitation

$NH_4(SO_4)_2$ saturation (%)	Protein (g L^{-1})	Protein Recovery (%)	Laccase (U L^{-1})	Laccase Recovery (%)	Sp. Act. of Precipitates (U g^{-1})	PF
0	0	0.0	0	0.0	0.00	0.0
10	0	0.0	0	0.0	0.00	0.0
20	0	0.0	0	0.0	0.00	0.0
30	0.11 (±0.02)	7.3	0	0.0	0.00	0.0
40	0.17 (±0.02)	12.0	0	0.0	0.00	0.0
50	0.21 (±0.02)	14.0	14.2 (±3.8)	5.5	67.6	0.4
60	0.24 (±0.02)	16.0	37.9 (±7.2)	14.8	157.9	0.9
70	0.28 (±0.03)	18.7	98.2 (±14.9)	38.2	350.7	2.0
80	0.15 (±0.04)	10.0	60.7 (±11.0)	23.6	404.7	2.4
90	0.08 (±0.01)	5.3	11.0 (±4.0)	4.3	137.5	0.8
100	0.03 (±0.01)	2.0	0.0	0.0	0.00	0.0
Total	1.27 (±0.14)	85.3	221.9 (±30.5)	86.4		

(± Standard deviation)
Sp. Act.: Specific activity; PF: Purification factor
Initial specific activity of crude samples: 170.4 ± 8.9 U g^{-1}

From the experiment, the first precipitated protein was observed at 30 % saturation with 7.3 % of protein recovery. Precipitated protein with laccase activity was only observed at 50 % saturation with laccase recovery of 5.5 %. The precipitated proteins at 30 and 40 % were the unwanted protein with high molecular weight. The distribution for the precipitated protein and laccase from the crude sample peaked at 70 % saturation with protein and laccase recovery of 18.7 and 38.2 %, respectively.

The total protein and laccase recovery from the protein fractionation via ammonium precipitation were 85.3 and 86.4 %, respectively. Thus, the loss of protein concentration and enzyme activity during the fractionation process were

less than 20 % of the initial sample. However, the highest specific activity of precipitated sample was observed at 80 % of ammonium saturation. The degree of purification was determined in the form of purification factor. From the fractionation in Table 4.20, the highest protein and laccase recovery did not increase the purification factor for each saturated fraction. The trend for the purification factor was seen to be increasing from the initial 0 % until 80 % saturation. The percentage of purification factor above 80 % saturation was lowered and became 0 factor at 100 % saturation. Since the fractionation is the first step in the whole process, the purity is considered before recovery. For that purpose, 80 % saturation was chosen for the next step in the partial purification process.

4.8.2 Void Volume Estimation in Sephadex Gel Filtration Column

Void volume is defined as volume of liquid in the interstitial space between grains in the bed. Since the study used the self-packed column, it is necessity to determine the void volume of a gel filtration column. The effect of peak broadening and sample dilution could be predicted from the void volume value. Experimental determination of void volume was done using Blue Dextran 2000 which has large molecular weight and completely excluded from gel beads. Figure 4.25 showed the chromatography curve of Blue Dextran 2000 in Sephadex G-25 and G-75 column.

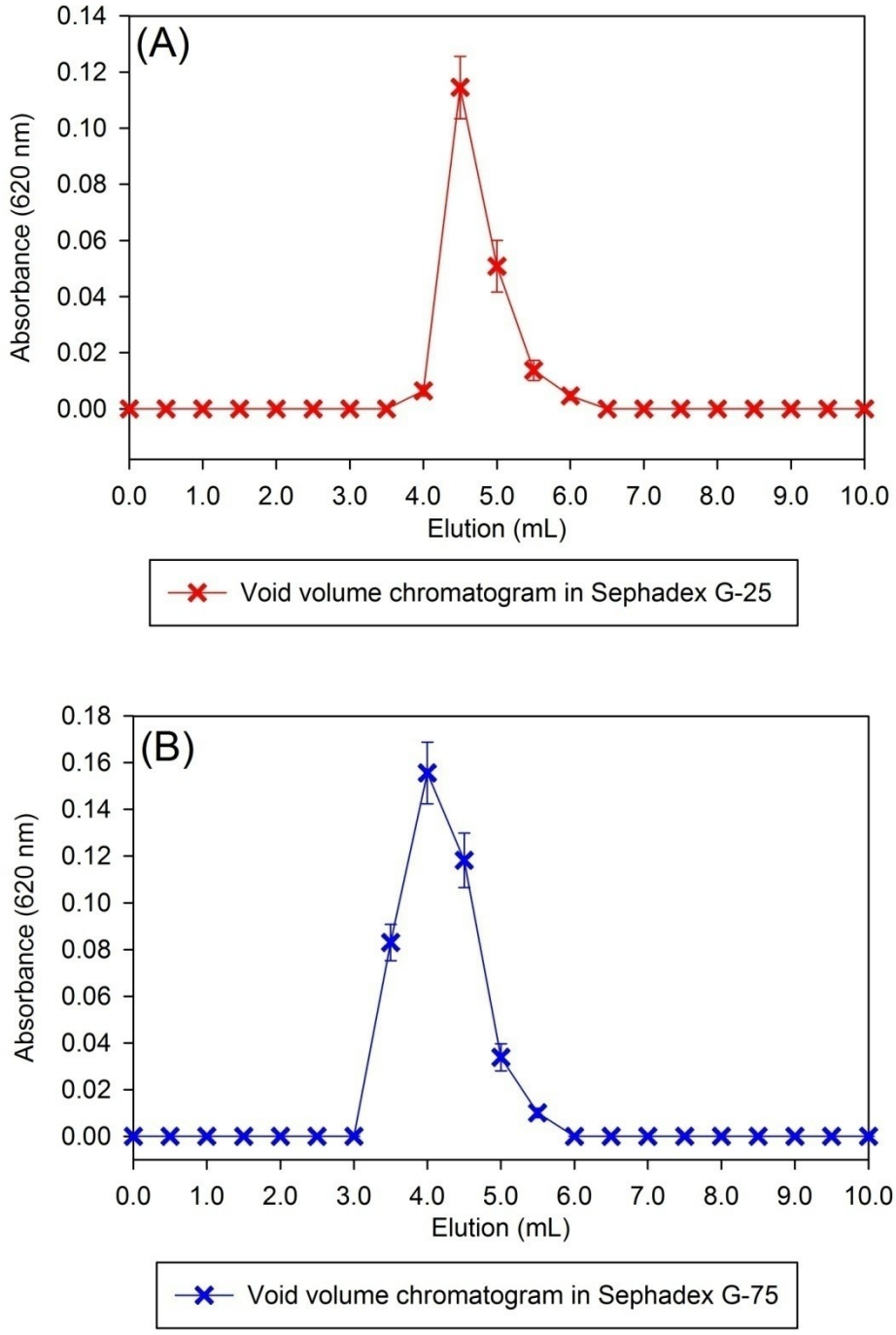


Figure 4.25: Void volume chromatogram: (A) Sephadex G-25 column and (B) Sephadex G-75 column (Error bar indicates standard deviation of 3 replicate experiments)

The eluent was collected from the time sample applied until the concentration of Blue Dextran reached its peaked. Based on the chromatogram, the peak for the column G-75 is slightly broad. However the G-25 column shows a

satisfactory symmetrical peak. The void volume values for the G-25 and G-75 column is shown in Table 4.21.

Table 4.21: Void volume (V_0) and total column volume (V_T) for self packed column

Sephadex Matrix	V_T (mL)	V_0 (mL)	V_X (mL)	Percentage of V_0 (%)
G-25	12.0	4.5	7.5	37.5
G-75	12.0	4.0	8.0	30.0

V_T: Total column volume; V_0: Void volume; V_X: Total gel matrix volume

The approximate percentage of void volume for a well packed column is 35 % of the total column volume (Dexter and Tanner, 1972). According to the supplier's instruction on the Sephadex gel matrix, the void volume for well packed column is equivalent to approximately 30 % of total column volume. The value of V_0 for the column G-25 and G-75 used in this study was similar to the volumes specified by (Dexter and Tanner, 1972) and the supplier's instruction. The actual value for void volume is depending on the packing materials, hydrodynamic pressure and method of packing (Fischer, 1980). Due to the inconsistent size and softness of beads between Sephadex G-25 and G-75, the void volume varies for the different types of matrix used. The void volume is used to determine the time for a protein sample to pass through the column as an eluent. If the protein sample is eluted after the total column volume elution, the sample may be bound to the gel matrix medium due to ionic interactions.

4.8.3 Laccase and Protein Gel Filtration using Sephadex G-25 and G-75

The partial purification of laccase was performed in two steps. The first involves the desalting of large molecular weight protein from entering the gel matrix pores. The impurities such as salts will permeate into the gel matrix pores whereas larger molecular weight such as proteins will be excluded first. In this study, Sephadex G-25 was used as gel matrix for the protein desalting due to the low molecular weight for fractionation of protein ranging from 1 to 5 kDa. With Sephadex G-25 as gel matrix, laccase is completely eluted from the column as laccase molecular weight is estimated about 60 to 80 kDa (Garcia *et al.*, 2006).

The chromatogram for laccase activity and protein concentration in the desalting chromatography (Sephadex G-25) is shown in Figure 4.26. In the current study, each fraction no. was equal to 0.5 mL of eluent. Based on the chromatogram, the protein content showed a broadening zone with low resolution. However, the laccase chromatogram is slightly narrow and symmetrical peak. For the protein profile, protein content was assayed for the eluent together with enzyme activity. Protein content for the eluent without enzyme activity was not determined. Increase in the ratio of volume of elution with laccase to volume of sample loading (V_E/V_{SL}) suggests that purified sample was diluted.

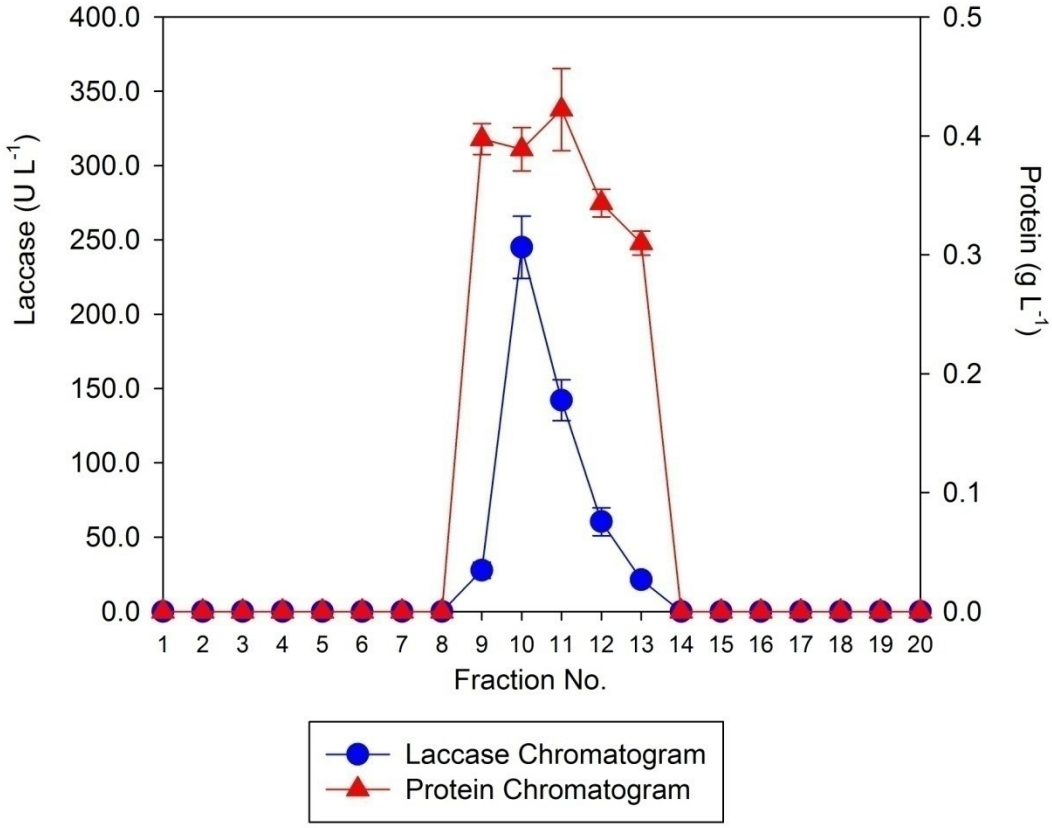


Figure 4.26: Chromatogram of laccase and protein for the samples from STR cultivation using Sephadex G-25 as gel matrix (± Standard deviation of 3 replicate experiments)

The maximum elution for laccase activity was observed in fraction no. 10. The initial sample volume loaded was 0.5 mL. A total of five fractions with elution volume of 2.5 mL showed laccase activity. Thus, the dilution factor accounted for laccase activity of sample in the column Sephadex G-25 was 5. The dilution effect on eluted sample was influenced by initial loading sample (mL) relative to the size of column. Large sample loading in small column volume may lower the fraction resolution and increased peak broadening of each eluent which indicates the dilution of sample was large. The crude sample was completely desalted when running in desalting column. This can be confirmed from the total eluent with laccase activity at 2.5 mL which was less than the calculated gel

matrix volume of 7.5 mL (Table 4.21). Thus, the total protein with laccase activity can be said to be relatively free from impurities such as salts, low molecular weight proteins, etc.

Partially purified sample from the desalting column was further purified by means of fractionation using Sephadex G-75 as gel matrix. The chromatogram of purified laccase activity and protein content using Sephadex G-75 is shown in Figure 4.27.

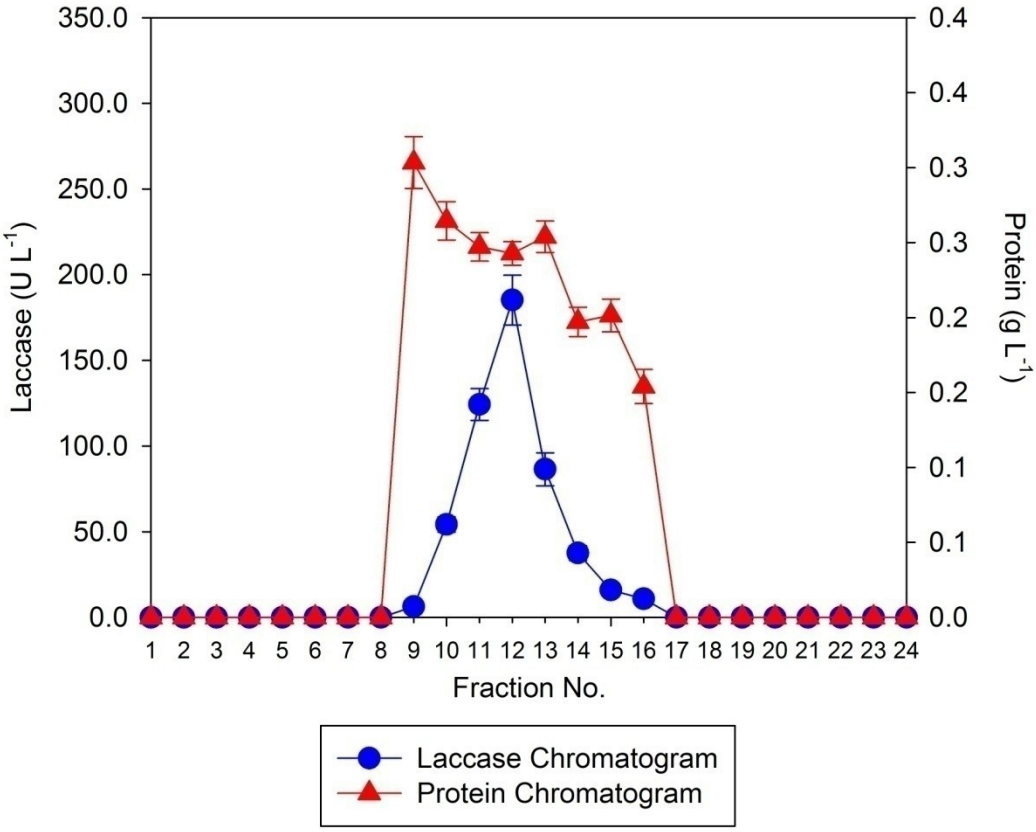


Figure 4.27: Chromatogram of laccase and protein for the samples from STR cultivation using Sephadex G-75 as gel matrix (Error bar indicates standard deviation of 3 replicate experiments)

The laccase chromatogram showed a fair resolution fractionation with symmetrical peak and short tailing peak which could be due to uneven sample

application. However the broadening peak of protein chromatogram showed low resolution fraction. Sadhasivam *et al.* (2008) observed the broadening peak in the protein elution profile with narrow peak of laccase profile while performing gel chromatography of laccase from *Trichoderma harzianum* using Sephadex G100 as gel matrix. The peak resolution depends on various factors including sample volume to column volume ratio, flow rate, column dimensions, packing density, methods of applying sample and gel matrix distribution.

The protein content was assayed only for the eluent with laccase activity. Maximum laccase activity was observed in fraction No. 12. About eight fractions with laccase activity were observed with the elution volume of 4.0 mL, which was less than total gel matrix volume (V_X) of 8.0 mL (Table 4.21). Therefore, the applied samples contained crude laccase in the Sephadex G-75 column were eluted in between the void volume and total column volume with laccase chromatogram peaked at 6.0 mL eluent. No laccase activity was observed from fraction no. between 17 and 24. Based on that, there was no indication of late elution of laccase from the Sephadex G-75 column. Thus, laccase crude sample fractionation was completely eluted from the column without any unusual interactions between the sample and gel matrices.

Ratio volume of elution with laccase to volume of sample loading (V_E/V_{SL}) was large which suggest that purified sample was diluted. Based on Equation 3.29 (Section 3.11.2), the calculated dilution factor (ratio volume of eluent with laccase to volume of sample loading) in Sephadex G-75 was 8 compared to Sephadex G-25 which was 5. The increase of dilution factor in G-75 column could be related to the water regain capability in Sephadex G-75 which is higher than the Sephadex G-25.

4.8.4 Laccase Activity and Protein Content Determination in Partial Purification Steps

Partial purification was performed in three steps which were precipitation, desalting and fractionation. Protein precipitation using ammonium sulfate was done in order to remove contaminants such as glucose, nutrients, salts etc. Protein precipitation was also done in order to concentrate the protein content in sample with low protein concentration. As discussed in Section 4.8.1, the optimum percentage of ammonium sulfate concentration for maximum purity of enzyme was 80%. The ammonium salt excess from precipitation experiment was removed in the desalting column. The calculated laccase activity and protein content in Sephadex G-25 and G-75 eluents were based on pooled samples.

Enzyme purity for each partial purification steps was summarized in Table 4.22. The purity of laccase crude sample precipitated using ammonium sulfate did not increase as purification fold obtained was 1.1. This could be the effect of ammonium salt excess which may be interfering with assay procedure for enzyme activity and protein content. The ammonium excess was removed by running the sample in desalting column that contains Sephadex G-25. Elution obtained from desalting column showed an increase in purity to 3.8. For purification using Sephadex G-75, protein fraction indicated the enzyme purity was 5.1 fold. Therefore the applications of ammonium precipitation and gel chromatography in purifying the enzyme increased purity up to 5 fold.

Table 4.22: Partial purification steps from STR cultivation by *P. sanguineus*

Steps	Volume (L)		DF	Lac. Act. ($U L^{-1}$)	Protein ($g L^{-1}$)	Sp. Act. ($U g^{-1}$)	Purification fold
	Initial	Final					
Crude	-	1	-	262.4 (±12.2)	1.68 (±0.07)	156.8 (±10.6)	1.0
Ammonium Precipitation	5.0 $x10^{-2}$	5.0 $x10^{-2}$	1	234.3 (±11.8)	1.33 (±0.08)	176.1 (±16.0)	1.1
Sephadex G-25*	5.0 $x10^{-4}$	2.5 $x10^{-3}$	5	242.2 (±10.7)	0.41 (±0.01)	590.9 (±21.7)	3.8
Sephadex G-75*	5.0 $x10^{-4}$	4.0 $x10^{-3}$	8	173.4 (±5.3)	0.22 (±0.02)	805.7 (±56.0)	5.1

DF: dilution factor; Lac. Act: laccase activity; Sp. Act.: specific activity.
Purification fold = (specific activity of each step) / (specific activity of crude sample)

4.9 SDS Gel Electrophoresis

Sample from the purification steps was loaded in gel electrophoresis. For molecular weight estimation, protein marker with wide range selectivity (Sigma) was loaded in the gel electrophoresis. Figure 4.28 showed the SDS PAGE analysis on partially purified sample from laccase fermentation. Three samples and a protein marker were loaded into the gel with S1 is the protein marker, S2 is commercial laccase from *Trametes versicolor* (Sigma), S3 is sample from stirred tank reactor (STR) cultivation and S4 is sample from the shake flasks cultivation. The commercial laccase from Sigma has molecular weight of 66 kDa.

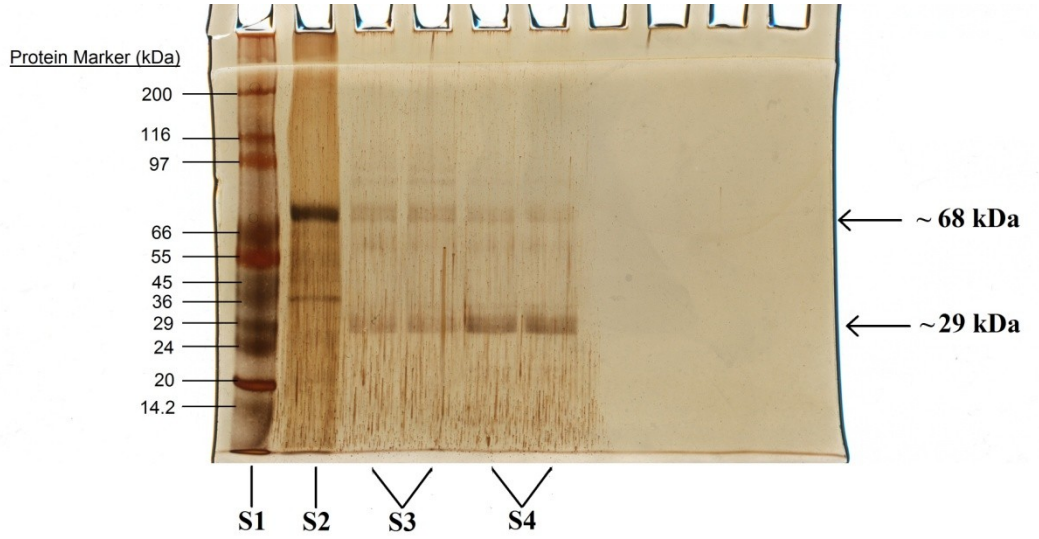


Figure 4.28: SDS PAGE (12 % gel strength, stained with silver staining method, (Heukeshoven and Dernick, 1985)) analysis on partially purified laccase from fermentation (S1: Protein marker (Sigma); S2: Commercial laccase from *T. versicolor* (Sigma); S3: Partial purified laccase from STR; S4: Crude laccase sample from shake flasks)

The protein bands seen in the commercial laccase preparation from Sigma (S2) and partially purified samples (S3 and S4) have similar molecular weight located within 66 to 97 kDa of the protein marker. This could be the protein band for the laccase enzyme as it was reported that the molecular weight of laccase protein was about 68 kDa (Vite-Vallejo *et al.*, 2009). Thus the visualized samples in the SDS PAGE may contain identical laccase protein when compared to commercial laccase from Sigma and also from previous studies (Vite-Vallejo *et al.*, 2009). Appearance of extra band at S3 and S4 at ~29 kDa suggested the presence of other unknown proteins which were eluted together with the probable laccase (~68 kDa)

The used of gel chromatography alone in the overall purification steps was not sufficient to obtain high purity laccase sample that should be indicated by the

presence of single protein band in the SDS PAGE (of 68 kDa). The application of gel chromatography in laccase purification is not specific and only based on the discrimination of molecular size. Samples have to be further purified using other chromatographic methods based on the interaction between laccase and gel matrix through the charge properties i.e. anion exchange chromatography (Bahrin *et al.*, 2010; Zhang *et al.*, 2010) and biorecognition i.e. affinity chromatography (Catalá *et al.*, 2011).

CHAPTER FIVE

5.0 CONCLUSIONS

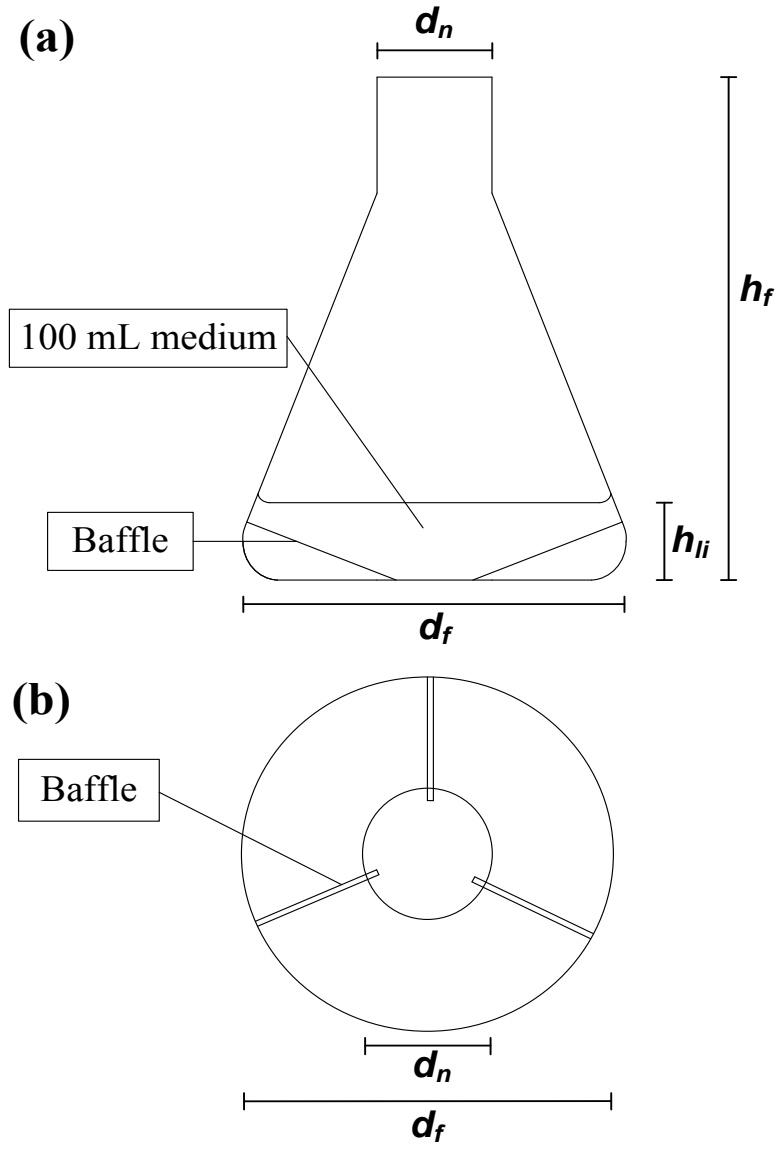
In this study, laccase production from *Pycnoporus sanguineus* was performed in shake flask and stirred tank reactor (STR). Cultivations were performed in batch mode in order to evaluate the effect of operating conditions on the overall fermentation process including fungal growth, glucose utilization and laccase production. Based on the study, it is concluded that:

1. The optimum conditions for maximum laccase production in STR cultivation were at agitation of 600 rpm, pH 4.43 and aeration 1.00 L min^{-1};
2. Optimization of selected operating variables in the STR indicated that pH effect was more pronounced than the effect of agitation and aeration rate;
3. The effect of baffled flask cultivation significantly influenced the kinetic parameters of fungal growth but not in laccase production by the fungus;
4. Higher amount of substrate in the STR cultivation was used for cell maintenance compared to the amount of glucose used for the growth activities by the fungus;
5. Laccase production was independent from the effects of different cultivation system;
6. Laccase production in shake flasks and STR were mainly observed during the reduced growth phase of the fungus which coincided with the depletion of glucose.

APPENDIX A

Schematic Diagram and Geometric Characteristic of Baffled Flask

Schematic diagram (a) Side view; (b) Top view:



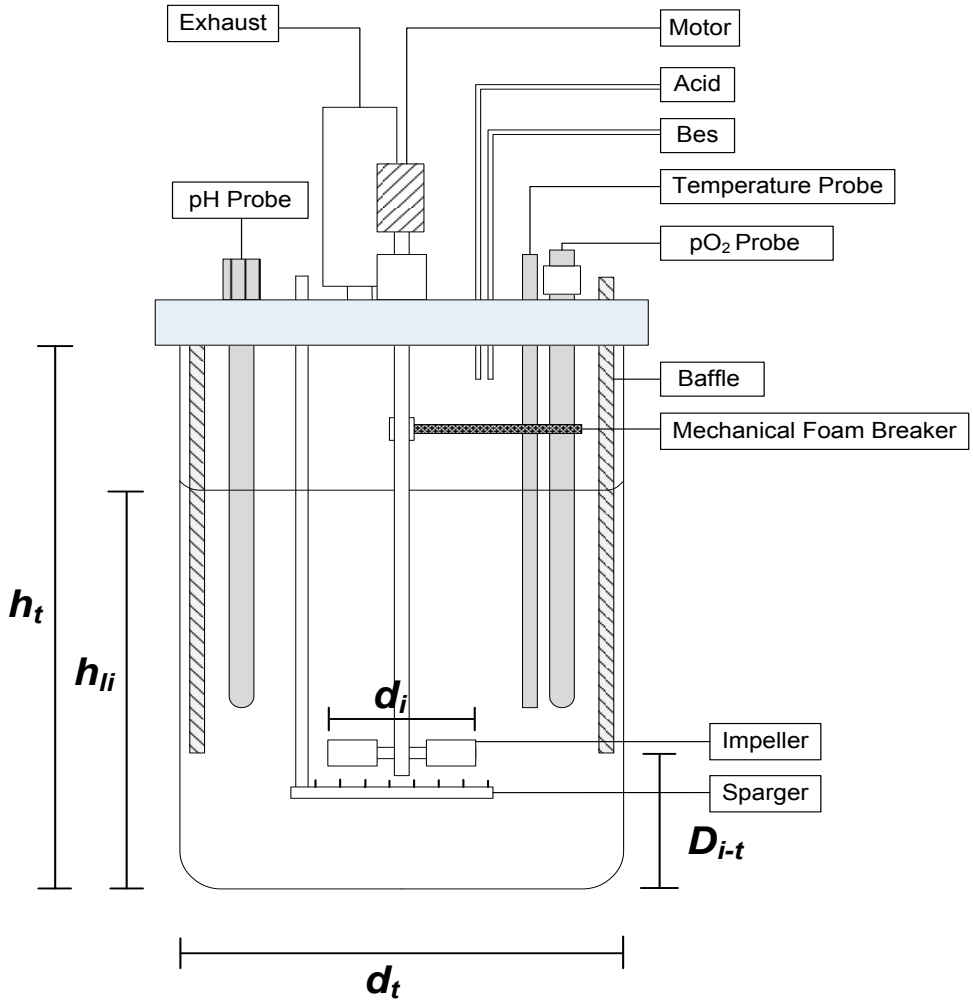
Geometric characteristics of the baffled flask:

Geometry	Specifications
Height of flask, h_f	145 mm
Diameter of the bottom, d_f	85 mm
Diameter of the neck, d_n	35 mm
Number of baffles	3
Total liquid volume	250 mL
Working volume	100 mL
Height of the liquid, h_{li}	~21 mm

APPENDIX B

Schematic Diagram and Geometric Characteristic of Fermenter

Schematic of fermenter setup:



Geometric characteristic of the fermenter:

Geometry	Specifications
Height of tank, h_t	240 mm
Diameter of inner tank, d_t	130 mm
Type of impeller	Rushton turbine
Diameter of impeller, d_i	52 mm
Width of impeller	10 mm
Sparger diameter	38 mm
Distance from impeller to bottom of the tank, d_{i-t}	52 mm
Number of sparger holes	14
Total tank volume	3.0 L
Maximum working volume	2.0 L
Initial working volume	1.3 L
Initial height of the liquid, h_{li}	~ 111 mm

APPENDIX C

Preparation of 50 mM Sodium Citrate Buffer pH 4.8

Sodium citrate (50 mM) pH 4.8 buffer was prepared by dissolving tri-sodium citrate and citric acid using ultrapure water. Henderson-Hasselbalch equation was applied in order to obtain pH of interest. Sodium citrate buffer was stored at 4 °C. Buffer calculation is shown below:

$$pH = pK_a + \log\frac{[conjugate\ base]}{[acid]} \qquad \text{(Equation A.1)}$$

or

$$pH = pK_a + \log\frac{[A^-]}{[HA]} \qquad \text{(Equation A.2)}$$

There are three acid dissociation constants (pK_a) for citric acid which are 3.13, 4.76 and 6.40. Thus, pK_a 4.76 was selected because it is close to the desired pH of 4.80. Variables in Equation A.2 are substituted with pH = 4.80 and pK_a = 4.76 to give:

$$4.80 = 4.76 + \log\frac{[A^-]}{HA} \qquad \text{(Equation A.3)}$$

Solve and rearrange Equation A.3:

$$\log\frac{[A^-]}{[HA]} = 0.04$$

$$1.0965 = \frac{[A^-]}{[HA]}$$

$$\frac{1.0965}{1} = \frac{[A^-]}{[HA]}$$

$$1.0965 : 1 = [A^-] : [HA] \qquad \text{(Equation A.4)}$$

From the ratio (Equation A.4), the total proportion is the sum of proportion for conjugate base [A^-] and acid [HA] which 2.0965. Thus, actual amount of conjugate base (tri-sodium citrate) and acid (citric acid) in 50 mM sodium citrate is as follow:

(i) Total amount conjugate base:

$$\frac{1.0965}{2.0965} \times 50 \text{ mM} = 0.0262 \text{ M} = \frac{0.0262 \text{ mol}}{1 \text{ L}} \times \frac{294.10 \text{ g}}{1 \text{ mol}} = 7.7054 \text{ g L}^{-1}$$

(ii) Total amount acid:

$$\frac{1}{2.0965} \times 50 \text{ mM} = 0.0238 \text{ M} = \frac{0.0238 \text{ mol}}{1 \text{ L}} \times \frac{192.13 \text{ g}}{1 \text{ mol}} = 4.5727 \text{ g L}^{-1}$$

Therefore, 7.7054 g of tri-sodium citrate and 4.5727 g of citric acid were dissolved in 1.0 L of ultrapure water.

APPENDIX D

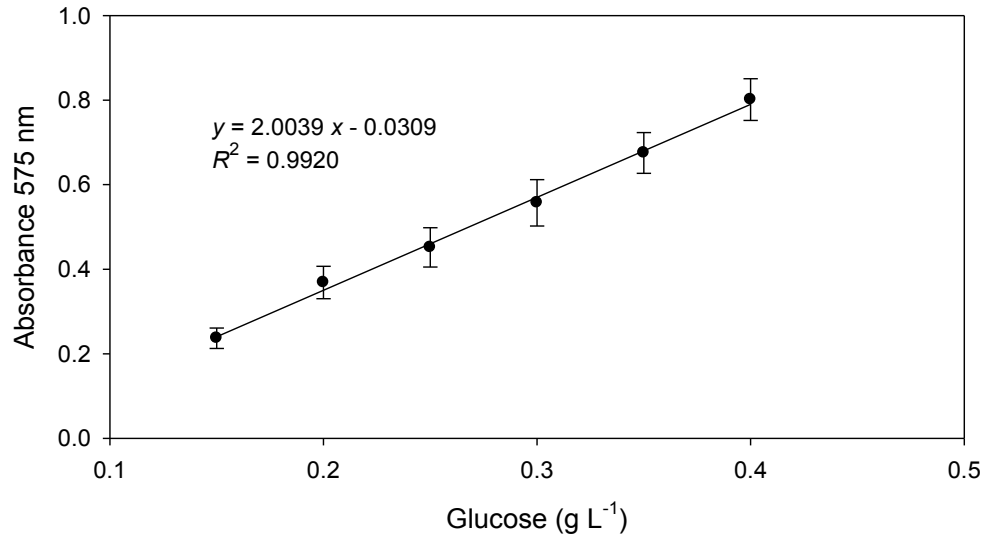
DNS Reagent Composition and Glucose Standard Calibration Plot

The composition of DNS reagent (Miller, 1959):

Chemicals	**(% w/v)**
3,5-Dinitrosalicylic acid (Sigma)	1.0
Phenol	0.2
Sodium hydroxide	40.0
Sodium sulphite*	0.05

*sodium sulphite were prepared separately and added into the reagent one hour prior to assay

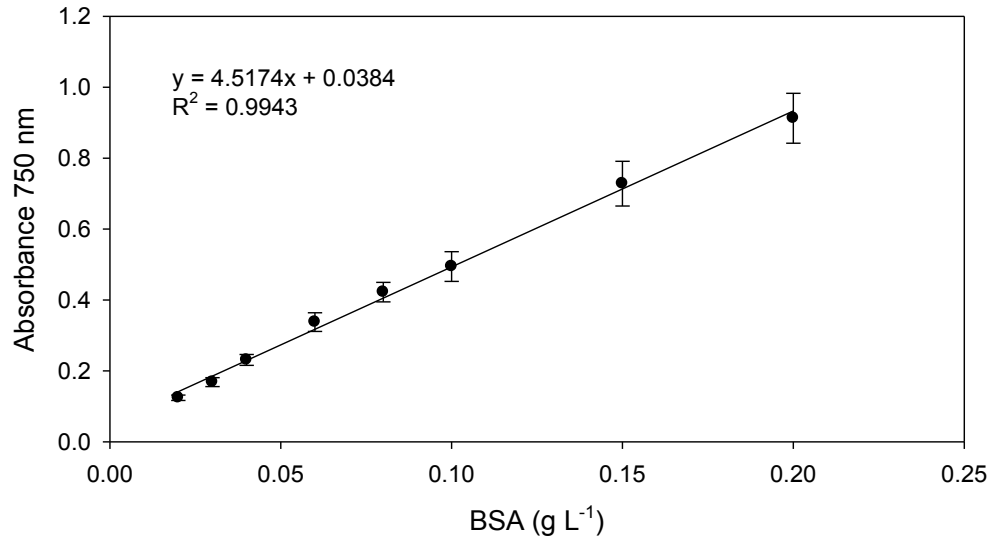
Glucose standard calibration plot (Error bar indicates standard deviation):



APPENDIX E

BSA Standard Calibration Plot

BSA standard calibration plot (Error bar indicates standard deviation):



APPENDIX F

Density and Molarity of Ammonium Sulfate

Density and molarity of ammonium sulphate at different temperature (Dawson *et al.*, 1986):

Temperature (°C)	**0**	**10**	**20**	**25**
Amount of $(NH_4)_2SO_4$ (g) to be added into 1 L water to give 100 % saturated solution	706.8	730.5	755.8	766.8
Amount of $(NH_4)_2SO_4$ (g) contained in 1 L 100 % saturated solution (G_{sat})	515.4	524.6	536.5	541.8
Molarity of saturated solution.	3.90	3.97	4.06	4.10
Density (g mL^{-1})	1.2428	1.2436	1.2447	1.2450
Specific volume in saturated solution (mL g^{-1}), (Sp. vol.)	0.5281	0.5357	0.5414	0.5435
Specific constant, P (Sp. vol. x G_{sat})/1000	0.2722	0.2810	0.2905	0.2945

APPENDIX G

Technical Data of Sephadex G-25 and G-75

Technical data of Sephadex G-25 and G-75 (adapted from GE Healthcare):

Technical specification	**G-25 (Medium)**	**G-75**
Working pH range	2 – 10	2 – 10
Particle size wet gel bead (μm)	85 - 260	90 - 280
Bed volume (ml g^{-1}) in distilled water	4 – 6	12 – 15
Fractionation Range (M_r) for globular proteins (kDa)	1 – 5	3 – 80
Max. flow rate (mL min^{-1})	*Darcy's Law	*Darcy's Law
Max. operator pressure (bar)	0.15	0.15

*The beads behave as rigid spheres obeying Darcy's Law

APPENDIX H

SDS PAGE Buffers and reagents (Laemmli, 1970)

List of stock solution for SDS PAGE buffers and reagents according to Laemmli (1970):

Buffers and Reagents	Components	Amounts	Final Volume
Acrylamide/Bis[a]	Acrylamide (Sigma)	29.2 g	100 mL
(30 % T, 2.67 % C)	N'N'-bis-methylene-acrylamide (Sigma)	0.8 g	
10 % (w/v) SDS	Sodium dodecyl sulphate, SDS (Sigma)	10 g	100 mL
1.5 M Tris-HCl, pH 8.8[b]	Tris base (Sigma)	18.15 g	100 mL
0.5 M Tris-HCl, pH 6.8[b]	Tris base (Sigma)	6 g	100 mL
Sample buffer[c]	0.5 M Tris-HCl, pH 6.8	1.25 mL	
(SDS reducing buffer)	Glycerol	2.5 mL	
	10 % (w/v) SDS	2.0 mL	9.5 mL
	0.5 % Bromophenol Blue.	0.2 mL	
	Ultrapure water	3.55 mL	
10X Electrode Buffer, pH 8.3[d]	Tris base	15.15 g	
	Glycine	72.0 g	500 mL
	SDS	10.0 g	
10 % (w/v) APS[e]	Ammonium persulphate (Systerm)	0.1 g	1.0 mL

All components were stored at 4 °C for 30 days unless otherwise mentioned.

[a] storage in dark

[b] pH adjustment using concentrated HCl (Systerm)

[c] storage at room temperature. 50 µL of β-mercaptoethanol was added to 950 µL sample buffer prior to use

[d] pH was not adjusted

[e] daily preparation

APPENDIX I

Preparation of 12 % Polyacrylamide Gel and Silver Staining Protocol

Formulation for 5 mL of 12 % of polyacrylamide gel for resolving and stacking:

Ultrapure water (mL)	30 % degassed Acrylamide/Bis (mL)	10 % SDS (mL)	Gel buffer (mL)		10 % APS (µL)		TEMED (µL)	
			Resolving (1.5 M Tris-HCl, pH 8.8)	Stacking (0.5 M Tris-HCl, pH 6.8)	Resolving	Stacking	Resolving	Stacking
1.7	2.0	0.05	1.25	1.25	25	25	2.5	5.0

Silver staining method according to Heukeshoven and Dernick (1985):

Step	Solution		Soaking time (min)
	Components	Final volume (mL)	
Fixing (2 x)	20 mL ethanol; 5 mL acetic acid.	50 x 2	15 x 2
Sensitizer*	15 mL ethanol; 3.4 g Na-acetate; 0.25 mL of 25% (w/v) of glutaraldehyde; 0.1 g $Na_2S_2O_3.5H_2O$	50	30 or overnight
Washing (3 x)	Ultrapure water	50 x 3	5 x 3
Silvering**	0.125 $AgNO_3$; 20 µL of 37% (v/v) formaldehyde	50	20
Developer	3.75 g Na_2CO_3	50	1
	20 µL of 37% (v/v) formaldehyde	50	3 to 7
Stopping	0.73 g EDTA-$Na_2.2H_2O$	50	10
Washing (3 x)	Ultrapure water	50 x 3	5 x 3
Preserving	5 mL of 87% (v/v) glycerol	50	30

*Na-acetate was dissolved in ultrapure water before ethanol was added. Glutaraldehyde and $Na_2S_2O_3.5H_2O$ was added prior to use.
**$AgNO_3$ was dissolved in water before the formaldehyde was added.

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LIST OF PUBLICATIONS

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