Faculty of Engineering & Science

Production and Characterisation of Lignin Peroxidase from Newly Isolated Thermophilic *Bacillus licheniformis* from Empty Fruit Bunch

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Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Abstract

With deployment of lignocellulosic material for biofuel application, effective lignin degradation will enhance removal of lignin. Biological pretreatment using thermophilic ligninolytic bacteria or enzyme can be exploited for lignin degradation due to their great tolerance at elevated temperatures. This research was initiated to screen for thermophilic ligninolytic bacteria native to empty fruit bunch (EFB) compost with the aim of isolating and identifying bacteria with relatively higher lignin degrading activity and carrying out a more in-depth study and evaluation on the production and characterisation of the key lignin degrading enzyme, namely lignin peroxidase, LiP from a newly isolated ligninolytic bacterium. Study was conducted to screen potential thermophilic ligninolytic bacteria from EFB compost using agar plate assay containing Methylene Blue (MB) or guaiacol and ligninolytic enzyme assay method. Ten ligninolytic thermophilic ligninolytic bacteria were successfully isolated and the most potent strain, *Bacillus licheniformis* CLMTCHB29 (Accession number:MH197076) was used in the subsequent production of LiP. In the production study, it was found that submerged fermentation is best suited for production of LiP that exhibited activity of 10.16 U/L. One factor at a time (OFAT) was used to determine the efficient range of 8 parameters for LiP production. The optimal condition for maximising LiP production were determined as follows: 50℃, pH 7, 16 % (v/v) inoculum size, 15 h incubation time, 0.4% (w/v) glucose, 0.2% (w/v) yeast extract concentration, 0.08% (w/v) kraft lignin concentration and 0.06% (w/v) $CaCl_2$. The LiP activity was found to be growth associated since the LiP production coincided with the active growth stage of *B. licheniformis*. Overall, 40% of increment in LiP activity (10 U/L to 14.30 U/L) was achieved following the individual effect parameter study. In the lignin degradation study, Fourier-Transform Infrared Spectroscopy (FTIR) analysis recorded 56.3% of degradation rate on the chemical bonding related to guaiacyl, syringyl and lignin subunit. The results suggest that *B. licheniformis* has a potentially significant role in lignin degradation process. Lastly, the LiP was partially purified using ultrafiltration and Biogel P-100 chromatography and as part of its characterisation study, enzyme kinetics, molecular weight and isoelectric point determinations, the effect of pH, temperature, metal ions and inhibitors on LiP were conducted.The purified LiP was revealed as a singl homogeneous band indicating its reasonably high purity on the SDS-PAGE with molecular weight of 26kDa and pI

value of 1.56. It has an optimum activity at pH 2.0 and highly stable over pH 5-9 with residual activity of above 50% after 10 h incubation. The Km and Vmax values with respect to VA and H_2O_2 were determined as 0.76 mM and 3.78 U mg^{-1}; and 80 mM and 15.96 U mg^{-1} respectively. This is the first report on thermotolerant LiP from *B. licheniformis*. It has an optimum temperature at 50℃ and retained its full activity following incubation at 60 ℃ and 70 ℃. The activity of partially purified LiP was stimulated in the presence of 1 mM Mn^{2+}, Ni^{2+}, Na^{2+} and Mg^{2+} by 34%, 27%, 12% and 11% respectively. However, it was highly inhibited by Fe^{2+} and Fe^{3+}. LiP also recorded residual activity of 78% and 89% on exposure to SDS and NAN_3 but no significant effect was observed on addition of 0.1 mM of EDTA and Tween 80. In conclusion, the finding on thermotolerant and alkali properties of LiP from novel *B. licheniformis* strain would present an opportunity for development of an effective bioconversion and valorisation of lignocellulosic materials.

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Nomenclature

ABTS	2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate)
AFEX	Ammonia Fibre Explosion
ANOVA	Analysis of Variance
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CBB	Coomassie Brilliant Blue
C:N	Carbon Nitrogen ratio
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
2,6-DMP	2,6-Dimethoxyphenol
DyP	Dye Peroxidase
EDTA	Ethylenediamine tetraacetic acid
EFB	Empty Fruit Bunch
EGTA	Ethylene glycol-bis(β-aminoether)-N,N,N',N'-tetraacetic acid
$FeCl_3$	Iron (III) chloride
$FeSO_4$	Iron (II) sulphate
FTIR	Fourier Transform Infrared Spectroscopy
GGE	Guaiacylglycerol-β-guaiacyl ether
KBr	Potassium Bromide
KCl	Potassium chloride
KCl-HCl	Hydrochloric acid Potassium Chloride Buffer
LAB	Lactic acid Bacteria
Lac	Laccase
LHW	Liquid Hot Water treatment
LiP	Lignin Peroxidase
MB	Methylene blue
MG	Malachite Green
$MgCl_2$	Magnesium chloride
$MnCl_2$	Manganese chloride
MnP	Manganese Peroxidase
MOF	Metal-Organic Framework
MSM-KL	Minimal Salt Medium with Kraft Lignin
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADH-DCIP	NADH-dependent 2,6-dichlorophenolindophenol
NaN_3	Sodium azide
$NiCl_2$	Nickel chloride

OD	Optical Density
OPFFB	Oil Palm Fresh Fruit Bunch
pI	Isoelectric point
rRNA	Ribosomal RNA
SDS	Sodium dodecyle sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SGB	Second generation bioethanol
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TSA	Tryptic Soy Agar
VA	Veratryl alcohol
WRF	White Rot Fungi
℃	Degree Celcius
gds	Gram of dry substrate
g/L	gram per Litre
h	hour
kDa	Kilo-Dalton
K_m	Michaelis-Menten constant (mol/l)
L	Litre
μL	Microlitre
μm	Micrometre
min	minutes
mg	milligram
ml	Millilitre
mM	Millimoles
nm	Nanometre
psi	pound per square inch
rpm	Revolution per minute
%(w/v)	weight by volume percent
%(v/v)	volume by volume percent
V	Initial reaction rate (mol/s)
V_{max}	Maximum reaction velocity (mol/s)
s	second
U/ IU	International unit for enzyme activity
ε	Extinction coefficient ($M^{-1}cm^{-1}$)
[S]	Substrates concentration (mol/l)
$\Delta A/min$	Average absorbance change per minute (min^{-1})
V_t	Total assay volume (ml)
V_s	Volume of sample (ml)
b	Light path (cm)

Chapter 1 Introduction

1.1 Lignin peroxidase

Lignin degradation using biological pretreatment is gradually increasing because of the high fuel demand and development of using greener bioenergy. Studies showed that lignocellulosic materials are underutilised whereby most of the biomass was used for incineration or landfill (Derman et al., 2018, Vaskan et al., 2018). These activities not only cause environmental pollution but also limit the utilisation of nutrient dense biomass that could offer good potential for conversion to high-value added products such as biofuel and biobased products (Suhartini et al., 2022). Though lignocellulosic material is a huge source of carbohydrate and lignin, its direct utilization as a feedstock for biofuel is hindered by their intricate structure. Thus, depolymerization of lignin in biomass into biofuel exhibits the major obstacle to techno-economic viability of the biomass biofuel-based technology (Medina et al., 2018).

Chemical, physical and physiochemical methods are effective in removing lignin but they are associated with high energy and chemical requirements, toxic- by product generation and undesired solubilization of hemicellulose (Baharuddin et al., 2013, Financie et al., 2016). The biological method in removing lignin presents the characteristic advantages of target-oriented lignin degradation by applying selective lignocellulosic microbial or enzymes system, thereby circumventing the generation of unwanted by-products or intermediates. Importantly, the biocatalytic process carried out under mild reaction condition that requires low energy input, cost and mild ecological influence (Baruah et al., 2018, Ferdeș et al., 2020). In biological pretreatment method, fungi are the most prominent lignin degrading microbial organisms capable of both selectively degrading lignin with high titre of ligninolytic enzyme production (Zabed et al., 2019). Ligninolytic enzymes (LiP, MnP and Lac) are known to target and break lignin linkages in the biomass or compounds that are structurally lignin-like such as environment polluting xenobiotics (endocrine-disrupting compounds, pesticides, dyes and chlorinated phenols) (Falade et al., 2018, Christian et al., 2005, Kathiravan and Gnanadoss, 2021). LiP has high redox potential that is responsible in degrading non-phenolic compound that is less likely to be

degraded by other ligninolytic enzymes. LiP are commonly found in fungi especially brown-rot fungi and white rot-fungi that would usually source from decayed wood that are capable of decaying or decompose lignin wood biomass (Zabed et al., 2019). However, fungi LiP has limited applicability because of weak adaptability and stability to pH and temperature. Moreover, poor culture condition such as oxygen limitation and high-risk contamination which hindered its application at industrial level (Masran et al., 2016). For example, the optimum temperature for a classical ligninolytic fungi, *Phanerochaete chrysosporium* has been reported as 30℃ (Zeng et al., 2013). This implies that fungi LiP is not suitable to be utilized in a reaction requiring a high temperature environment as required typically in composting process, paper and pulp mill effluent.

Recently, bacteria and actinomycetes also possess the ability in degrading lignin and aromatic substances. Bacteria were found to play an important role in biogeochemical cycling of lignin containing woody biomass in diverse ecosystems such as compost, soil, and rumen of animals, sediments, guts of insects and aquatic and terrestrials habitats (Levy-Booth et al., 2021, Li et al., 2020, Díaz-García et al., 2020). Thermophilic bacteria are harnessed for their capability to produce thermostable ligninolytic enzymes that may degrade lignocellulolytic biomass and pose low risk of contamination (Bhalla et al., 2013). Moreover, the main reason in selecting thermostable enzymes in bioprocessing or biorefineries of lignin is due to the intrinsic thermostability, which implies possibilities for prolonged storage, low activity losses during processing even at elevated temperatures during raw material pretreatment (Kumar et al., 2021). Thermostable enzyme that performs at high temperature often promotes better enzyme penetration and cell wall disruption of the biomass which leads to low enzyme consumption and more cost effectiveness (Turner et al., 2007).

1.2 Research Gap

The growing concern of enzymatic lignin degradation has received increasing attention due to its high selectivity and energy efficiency when compared to other pretreatment process such as uncatalyzed steam explosion, acid pretreatment and alkaline pretreatment. Extensive studies have focused on lignin degradation and isolation of ligninolytic enzyme by fungi since fungi are well-equipped with lignin

degradability and high enzyme production (Chang et al., 2012, Piñeros-Castro and Velásquez-Lozano, 2014, Hamisan et al., 2009). However, low thermotolerant properties and acidic working pH condition have hindered the application of fungi LiP in industrial lignin degradation process which often involves high temperature and alkaline working condition. It was reported that bacteria occupy a huge population during the composting process under thermophilic range. Indeed, studies indicated that thermophilic bacteria with ligninolytic ability occupy a huge population in the early stage of the EFB composting process (Ahmad et al., 2011b). Hence, ligninolytic bacteria in bioceonversion of lignin receive attention for the past two decades. Riyadi et al. (2022) revealed that three indigenous thermophilic bacteria isolated from EFB compost such as *Stenotrophomonas* sp., *Bacillus subtili*s, and *Aeribacillus* sp. demonstrated ligninolytic enzyme activities towards LiP, MnP and Lac (Riyadi et al., 2022). These thermophilic bacteria that are present in EFB compost may be potentially useful in lignin degradation. However, enzymatic degradation from thermophilic bacteria is still underexplored. Moreover, the major drawback of bacterial lignin degradation is the low yield of ligninolytic enzymes. There is the necessity to isolate novel thermophilic bacteria that exhibit ligninolytic enzyme from EFB compost and examine the nutritional and physicals factors that could enhance the LiP production. Thermophilic bacteria are expected to secrete enzymes with better stability and resistivity to denaturation than the mesophiles, which require further investigation. Thus, a preliminary evaluation on the degrading ability of their enzymes and its characteristics such as temperature stability, substrate specificity, tolerance towards metal ions and catalytic properties is an important pre-requisite in examining its suitability for industrial use.

1.3 Objectives

This study was initiated to discover, produce and characterise novel thermotolerant and alkali-tolerant LiP from thermophilic bacteria that is potentially useful in lignin degradation process. The main objectives of this study are as shown below.

1. To screen, isolate and identify the thermophilic ligninolytic enzyme producing bacteria from EFB compost.

2. To evaluate productivity of ligninolytic enzymes in Submerged Fermentation (SmF) and Solid-state fermentation (SSF) process the delignification ability of the isolated bacteria under fermentation process.
3. To determine optimal growth medium concentration and conditions for the isolated bacteria in enhancing production of ligninolytic enzyme.
4. To purify and characterise the extracellular LiP enzyme from the newly isolated thermophilic *Bacillus licheniformis* strain.

1.4 Scope of work

1. Isolation and screening of thermophilic bacteria which produce lignin degrading enzymes from EFB compost. Young EFB compost was re-enriched in MSM-KL medium and screened with MSM-KL solid plate assay containing MB, guaiacol under incubation of 50℃.
2. Study the lignin degrading enzyme profile from the selected strain and determine the dominant lignin degrading enzymes. Bacteria that showed good growth and decolourisation on solid plate assay were selected and incubated in MSM-KL medium at 50℃ under submerged fermentation to determine the ligninolytic enzyme activity (LiP, MnP, Lac) using standard enzyme assay method.
3. Identification of the potent bacteria. The most potent bacterium with the highest LiP activity was sent to NextGene for identification and its genomic sequence was deposited in the GenBank.
4. Determine optimal cultural and nutritional parameters for enhanced production of LiP from *Bacillus licheniformis*. The studied cultural parameters including fermentation configuration, incubation time, inoculum size, temperature, pH. The studies on nutritional parameters included the effect of glucose, yeast extract, lignin and $CaCl_2$ concentration on LiP production.
5. Determine the kraft lignin degradability of *Bacillus licheniformis* through FTIR analysis or functional group of kraft lignin structure.
6. In purification of LiP from *B. licheniformis*, SDS-PAGE of the partially purified enzyme was conducted to identify the molecular weight, and to check the purity of the protein, including the determination of its pI value.

7. In the enzyme characterisation and kinetic studies, the optimum pH and temperature, and stability of the LiP were investigated, including the influence of metal ion inhibitors and catalytic parameters, namely the Km and Vmax values of the enzyme were determined using the partially purified LiP. Several metal ions reported to be involved in biological function were evaluated for their influence in the activity of purified ligninolytic enzyme.

1.5 Research significance

The novelty of this study is in screening of newly isolated ligninolytic thermophilic bacteria which can produce LiP that exhibits high temperature stability and resistance. This would be significantly useful in lignin degradation processes such as black liquor degradation, lignocellulosic composting process, biofuel production and paper and pulp mill effluent at elevated temperature. Moreover, the alkaliphilic properties of LiP enable it to work at neutral or alkaline condition which is extremely useful for application such as biopulping and dye decolourisation industry.

Chapter 2 Literature Review

2.1 Introduction

Malaysia and Indonesia are the major producers and exporters of palm oil in the world (Prokurat, 2013). The worldwide production of palm oil increased from 52.6 million tons in 2011 to 61,7 Mt in 2015. This is expected to grow, mainly due to the growing demand of palm oil in broad range of use in the food industry, palm-based biodiesel and the socio-economic benefits production in rural areas (Gilbert, 2012). However, the rapid growth in oil palm industries, a huge amount of oil palm biomass residues especially after harvesting of oil palm fruits, replantation of trees and oil palm processing. Oil palm industry generate about 47% of oil palm biomass waste. Palm biomass waste included empty fruit bunch (EFB), fronds, mesocarp fiber (MF), trunk, shell and palm kernel (Sumathi et al., 2008). As shown in table 2.1, EFB is the highest oil palm biomass generated from oil palm industries which constituted about 15.8-17.0 million tons that is potentially available per year (Derman et al., 2018). EFB is often considered a waste product that requires proper utilization (incineration) and involves disposal costs. Proper disposal of EFB becomes a big challenge for the producers of palm oil and a sustainable solution could significantly improve the palm oil industry profitability and environmental protection (Vaskan et al., 2018, Derman et al., 2018).

Table 2.1 Types and quantities of oil palm biomass produced in Malaysia (Derman et al., 2018)

Types of oil palm products	Quantity/year (Million tons)
Empty fruit bunches (EFB)	15.8- 17.0
Fronds	12.9
Mesocarp fiber (MF)	9.6
Trunk	8.2
Shell	4.7-5.9
Palm Kernel	2.11

In the current prospect, EFB is used as a mulch or fertiliser in palm oil plantation or other agriculture fields due to its high potassium content (Lim and Lee,

2012). However, its application area as fertiliser is rather limited and it is hardly to be transport and decompose. EFB also used as a fuel for the purpose of heat and electricity generation (Hosseini and Wahid, 2014). This process involves drying, shredding, and pressing of EFB into convenient briquette that are easy to distribute and burn. However, the economic and environmental concern of such process is still doubtful mainly because large moisture content in EFB and incineration caused pollution (Yusoff, 2006). In year 1999, Malaysia government has introduced the 5th Fuel Diversification Policy (FDP) by including biomass renewable energy as the fifth energy resources to sustain future energy demand (Mekhilef et al., 2011). In line with the launch of FDP, biochemical conversion of EFB into biofuel and high value-added products would be another sustainable alternative in handling the EFB. For example, the increase in world production of fuel ethanol from 50 to 93 billion L/y between 2007 and 2014 (Vaskan et al., 2018). Lignocellulosic material is a probable resource for bioethanol production.

2.2 Oil Palm Empty Fruit Bunch (EFB)

EFB are one of the major lignocellulosic biomasses which consists of 3 major components, namely cellulose, hemicellulose and lignin. Cellulose content of oil palm EFB is 37.3-46.5%, while hemicellulose and lignin content are 25.3 – 33.8% and 27,6 -32.5% respectively (Syafwina et al., 2002). Cellulose is the polymers of C5 and C6 sugars and it is the key component to produce bioethanol. The high cellulose content in EFB which in turn, can serve as feedstock for bioethanol production (Sudiyani et al., 2013).

The bioethanol produced from EFB, are known as second generation bioethanol (SGB). Bioethanol is primarily produced from edible resources such as sugars and starchy material (corn, sweet potato). Bioethanol that is produced from starchy materials is known as First Generation Bioethanol (FGB) (Limayem and Ricke, 2012). However, the ethical concern on using food as a fuel resource is the main drawback of FGB production. Considering the ethical issue with food, EFB can be a more promising feedstock for bioethanol production since they are abundantly available from palm oil wastes and limited competition with food source. It is estimated that 0,608 tons of ethanol would be produced from EFB if it is being fully

utilized based on the annual production of 3.66 tons per hectares of palm oil plantation reported in year 2016 (Derman et al., 2018). This may contribute to the decrease of imports gasoline through partial substitution of this fossil fuel and reduce carbon emission. Apart from that, the characteristic of low cetane and high octane make bioethanol produced from oil palm EFB a more sustainable option in replacing blend with gasoline (Suhartini et al., 2022). Several studies highlighted the feasibility of conversion of EFB to bioethanol in laboratory scale and pilot scale. Huailuek et al. (2019) and Medina et al. (2018) also claimed that valorisation of EFB via bioconversion approach could offer multiple opportunities to improve economic and environmental sustainability (Huailuek et al., 2019, Medina et al., 2018). Owing to the recalcitrance of the lignin structure, pretreatment of EFB is required to remove the lignin and reduce the crystallinity of cellulosic material so that cellulose more amenable to the action of enzyme in the hydrolysis process (Fig. 2.1). The conversion efficiency of EFB to bioethanol is reported to be between 13.68 to 14.5% per raw EFB (Han et al., 2011, Gupta and Verma, 2015). Low bioethanol yield from EFB is related to low efficacy of lignin removal in the pretreatment method, which can hinder the hydrolysis phase of conversion thus reducing the efficiency of bioethanol conversion. Therefore, an efficient bioconversion or pretreatment to depolymerise the lignin structure of EFB is necessary to ensure that future bioethanol production from EFB is commercially and environmentally sustainable.

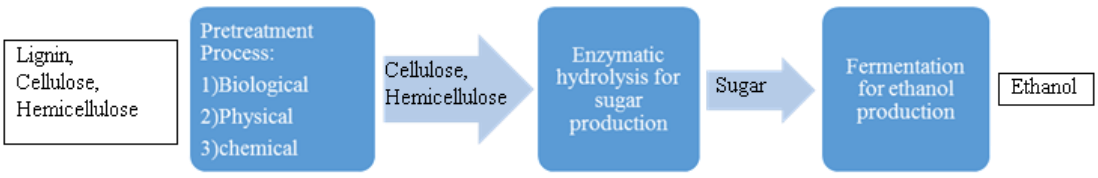


Figure 2.1 Second Generation Bioethanol (SGB) process (Aditiya et al., 2016)

2.3 Pretreatment process

Pre-treatment process is the critical step in the SGB production from lignocellulosic material as it has large impact on the conversion of cellulose and it strongly affect the downstream costs (Sindhu et al., 2016). In EFB, the cellulose is surrounded by lignin and hemicelluloses. An effective pretreatment can change the lignin and hemicellulose

structure to increase substrate porosity with lignin redistribution which enables maximal exposure of cellulose surface area in the enzymatic hydrolysis. This could further lead to greater conversion of cellulose to monomeric sugar and higher fermentable sugars production (Sindhu et al., 2016, Limayem and Ricke, 2012). During the depolymerisation of lignin, variety of toxic by-products (hydroxymethylfurfural, furfural and phenolics) are formed which could hinder the subsequent enzymatic hydrolysis and fermentation process in the operation and economical aspect. Thus, an ideal pretreatment process should acquire minimum formation of inhibitory compounds. Overall, yield of sugars, formation of inhibitors and chemical and energy consumptions are the primary factors in evaluating a pretreatment efficiency and cost effectiveness in a pretreatment process (Wyman et al., 2005).

In general, there are several pretreatment technologies for lignocellulose biomass including physical, chemical and biological pretreatment. Each of these methods requires different energy demand, chemical requirement, and operational configuration.

2.3.1 Physical Pretreatment

In physical pretreatment technology, it mainly consists of Uncatalyzed Steam Explosion and Liquid Hot Water treatment (LHW) (Zheng et al., 2009, Agbor et al., 2011). Physical pretreatment process is often used to reduce the particle size. In order to increase the total surface area and reduce the crystallinity of cellulose structure for enzymatic attack. Uncatalyzed Steam Explosion utilized high pressure saturated steam whereas LHW uses high temperature water to promote hemicellulose hydrolysis.

In Uncatalyzed Steam Explosion, high-pressure saturated steam is used to heat up biomass rapidly at high temperature of about 160-240℃ for several minutes and then released to terminate the pretreatment process (Agbor et al., 2011). During swift release of pressure, biomass experienced an explosive decompression. Throughout this whole process, acetic acid is released by biomass and often used to hydrolyse the hemicellulose to produce glucose and xylose monomer. From the description of action mode above, Uncatalyzed Steam Explosion do not require any addition of chemicals and it can be operated directly after the fruit has been stripped off from the palm oil

production. However, uncatalyzed steam explosion has low hemicellulose sugar yield with partial destruction of lignin carbohydrate. In addition, high temperature condition causes generation of toxic compounds or fermentation inhibitors which might hinder the following hydrolysis and fermentation process in ethanol production (Alvira et al., 2010). Apart from that, it requires a high energy input and this might increase the operational cost at commercial scale.

Liquid hot water pretreatment has similar action mode as uncatalyzed steam explosion, the only difference is using liquid hot water instead of high-pressure saturated steam. Hot water is used to break down hemiacetal linkages and acids are liberated during biomass hydrolysis which is useful in breaking down ether linkages in biomass. In this pretreatment, lower temperature of hot water is utilized which ranged from 160-190℃ as compared to uncatalyzed steam explosion (Agbor et al., 2011). In addition, no neutralization process is required since water is the only solvent involved. However, huge amount of hot water will result in low concentration of monomeric sugars and high energy demand for heating purpose in downstream process (Alvira et al., 2010). High cost due to high energy demand in physical process is the main drawback that it is not applicable in full-scale process.

2.3.2 Chemical Pretreatment

As for chemical pretreatment, acid pretreatment, alkali pretreatment and Ammonia Fibre Explosion (AFEX) are the three commonly used pretreatment methods. In acid and alkali pretreatment processes, both employ dilute acid and bases respectively to solubilise hemicellulose (Zheng et al., 2009). Usually sulphuric acid, hydrochloric acid, nitric acid, phosphoric acid and peracetic acid are used in acid pretreatment process whereas the common bases include sodium hydroxide and calcium hydroxide. In dilute acid pretreatment, there are two types of configurations available for aqueous solution of substrate and solid biomass respectively. In the first configuration, dilute acid is preheated to desired temperature before added into aqueous solution of substrate. As for second configuration, biomass is physically pretreated with wire mesh and submerged in a circulating bath of dilute acid. This bath of dilute acid is heated to desired temperature which is dependent on the severities of pretreatment effect. Greater severities of pretreatment effect increase with increasing temperature. In both of these pretreatment configurations, hemicellulose hydrolysis

start upon the addition of acid or acid contacting with biomass or substrate (Agbor et al., 2011). Although chemical pretreatment processes are able to diminish most of the lignin content, it requires large energy supply. Apart from that, an extra neutralisation process is needed to remove the acids or bases prior to enzymatic hydrolysis process and this leads to greater capital cost. Additionally, formation of inhibitors such as (furan derivative, phenol derivatives) has hindered the enzymatic hydrolysis process (Talebnia et al., 2010, Behera et al., 2014).

2.3.1 Biological Pretreatment

Lastly, biological pretreatment utilizes microorganism or enzymes to degrade lignin and hemicellulose. Lignin degrading microorganism or enzymes were used to degrade the lignin content prior to enzymatic hydrolysis in bioethanol production. In the current prospect, microbial pretreatment and enzymatic pretreatment are the commonly used biological method in pre-treating lignocellulose biomass (Bahena-Molina et al., 2022). These two methods utilise ligninolytic enzymes to digest and degrade lignin. Generally, biological pretreatment is a more environmentally friendly pretreatment method in enhancing biodegradation of lignocellulosic biomass since no chemicals are required. Additionally, it is more cost effective as it does not require high energy supply and high chemical requirement condition. Moreover, biological pretreatment process does not produce toxic compounds as much as physical and chemical process (Agbor et al., 2011, Alvira et al., 2010) and thus less inhibiting effect on microbial growth and enzyme accessibility. Despite some advantages, the long pretreatment period and low downstream yield are major drawbacks which are not techno-economically sustainable for lignin degradation at industrial level (Zabed et al., 2019, Masran et al., 2016). Hence, exploitation of thermophilic ligninolytic bacteria could bring a breakthrough for effective lignin degradation.

2.3.1.1 Single strain pretreatment

Microbial pretreatment is commonly done by employing single culture of fungi, bacteria or microbial consortia system that produce secrete one or more extracellular ligninolytic enzymes (LiP, MnP and Lac) which are responsible for the degradation of aromatic polymer and aliphatic fragments (Baruah et al., 2018). In single strain

pretreatment, it utilises single strain of fungi or bacteria cells for the degradation via fermentation process by using lignocellulose biomass as the substrate. In general, the lignin decomposing fungi are classified into white rot, brown rot and soft rot fungi (WRF, BRF and SRF). Among these fungi, WRF and BRF are renowned for their lignin degrading capability that have long been used for the pretreatment of lignocellulose. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Pleurotus streatus*, *Aspergillus niger*, *Trichoderma reesei* and *Trametes versicolor* are commonly employed in for ligninolytic pretreatment due to their capability in producing high titre of ligninolytic enzymes in metabolizing lignin (Kumar and Sharma, 2017, Shirkavand et al., 2016, Sánchez, 2009). However, this pretreatment process requires long incubation time that may take up from weeks to months (Zabed et al., 2019). Moreover, fungi has low stability in practical treatment under extreme environmental and substrate conditions.

Utilization of bacteria in pretreatment could be more promising for reducing the pretreatment time as the growth rate and metabolic activity of bacteria are faster than those fungal species (Zabed et al., 2019). Lignin decomposing bacteria particularly include actinomycetes, proteobacteria and firmicutes. For instance, some bacteria such as *Rhodococcus* sp., *Pseudomonas* sp., *Sphingomonas* sp. and *Bacillus* sp. can secrete ligninolytic enzymes such as LiP, MnP and Lac (Kamimura et al., 2019, Rahmanpour and Bugg, 2015, Masai et al., 2007, Ragauskas et al., 2014). Morii et al. (1995) claimed that bacteria isolated from compost soil such as *Azotobacter*, *Bacillus megatarium* and *Serratia marcescens* are capable of decolorizing or solubilizing lignin. The capability of lignin degradation was correlated with the activity of laccase (Morii et al., 1995). In Raj et al. (2006) study, *Aneurinibacillus aneurinilyticuscan* was reported to degrade lignin to low molecular weight aromatic compounds such as guaiacol, acetoguaiacone, gallic acid and ferulic acid (Raj et al., 2006). In addition, it has been shown that bacteria isolated from termite gut also has lignin degradation capability (Azizi-Shotorkhoft et al., 2016, Ayeronfe et al., 2019, Anukam et al., 2020). These studies confirmed that bacteria contribute to lignin degradation.

Although lignin degradation by the bacterial strains is not as potent as offered by fungal species due to low ligninolytic enzyme production bacteria have relatively greater environmental tolerance in terms of alkalinity and thermostability, which make

it possible for large-scale cultivation and production (Xu et al., 2021, Wang et al., 2013b). Therefore, study on lignin degrading bacteria is necessary.

2.3.1.2 Microbial consortium pretreatment

Microbial consortia consist of mixed culture of fungi or bacteria that work synergistically on the biomass during pretreatment. In fact, microorganisms in nature live in community and carry out synergistic metabolic activities to degrade complex substrates, which are often difficult to decompose by single strain culture (Zabed et al., 2019). Thus, it has relatively shorter pretreatment time than fungal pretreatment at about several days. Various studies have shown microbial consortium produce more active lignocellulosic enzyme complexes than single strain culture due to their higher stability and adaptability to environmental conditions such as temperature, pH and inhibitors (Ali et al., 2020, Kong et al., 2018). Kong et al. (2018) reported 48.6% of lignin degradation in wheat straw by microbial consortium TC-5 via anaerobic fermentation. Similarly, Xu et al. (2021) also demonstrated 31.18% of lignin degradation in rice straw by lignin-degrading bacteria consortium in 7 days of incubation in mineral salt. However, maintenance of the effective conditions for synergistic ligninolytic activities of the microbe's population is the main challenges of employing microbial consortia in real-world application. Apart from that, the knowledge on the functional microbial and the synergistic metabolic mechanisms is rather limited, which hinders the application of microbial consortium in lignin degradation. In depth study is required to dissect multi-layer complexities of microbial, key enzymes and synergistic mechanisms via omics technology (Xu et al., 2021). Furthermore, some of the pretreatment microorganisms could produce cellulase and xylanase enzymes other than ligninolytic enzymes. Hence, part of cellulose and hemicellulose were also degraded and digested by the pretreatment microorganism, which results in a huge reduction in the net quantity of total soluble sugar produced in the enzymatic hydrolysis process (Wang et al., 2013a).

2.3.1.3 Enzymatic pretreatment

In enzymatic pretreatment process, it usually employs crude or purified or partially purified enzymes derived from the ligninolytic microorganisms to pretreat the biomass. Crude enzyme cocktails could be more promising for enzymatic pretreatment

in term of techno-economic issues. Crude enzymes can be produced and collected easily by fungi and bacteria capable of producing extracellular ligninolytic enzymes. Bilal and Iqbal (2020) reported the presence of other ligninolytic auxiliary enzymes and mediators such as feruloyl esterase may facilitate the ligninolytic enzymes for effective lignin degradation by cleaving the diferulic bridges between xylan chains and thus opening then structure and liberating the lignin molecules. Although the crude enzyme is predominant by ligninolytic enzyme, the presence of some cellulolytic enzyme facilitates the hydrolysis of cellulose material simultaneously that can save energy and time (Bilal and Iqbal, 2020). Moreover, it also overcome the disadvantage of microbial pretreatment on sugar loss in the pretreatment process. Numerous studies demonstrated similar amounts of lignin as achieved in microbial pretreatment at shorter incubation time. Ligninolytic enzyme extracted from *Ganoderma lucidum* immobilized in alginate chitosan beads showed 50.23-54.1% lignin removal in corn stover, cotton stalk, sorghum stover and sugarcane bagasse. Silva et al. (2014) and Mukhopadhyay and Banerjee (2015) also reported effective degradation using MnP and Lac single pretreatment respectively on coconut shell, sugarcane bagasse, sisal fiber and bambusa bamboo (Silva et al., 2014, Mukhopadhyay and Banerjee, 2015). These studies had proven the effectiveness of crude enzyme treatment on lignocellulosic material. To be techno- eco effective, the characteristics, stability and degradation mechanisms of an enzyme is critical in determining the potential of enzyme in industrial application.

The advantages and disadvantages of physical, chemical and biological pretreatment are shown in table 2.2 below. Biological method is favoured since other methods hinder the fermentation process for ethanol production due to generation of toxic compounds or fermentation inhibitors. In addition, other pretreatment methods such as alkali pretreatment method requires high capital cost. In fact, biological method is a natural phenomenon that mimics the degradation of agriculture biomass in the presence of microorganism or enzymes. Thus, this method is considered as the most environmentally friendly approach since it requires mild environment conditions.

Table 2.2 Lignocellulose biomass pretreatment methods (Bahena-Molina et al., 2022)

Method	Advantages	Disadvantages
Uncatalyzed Steam Explosion	• Low usage of chemicals • Low energy input • Low capital investment	• Low Hemicellulose sugar yield • Partial destruction of lignin carbohydrate • Generation of toxic compounds
Liquid Hot Water (LHW)	• No addition of chemical • Low capital cost	• Low concentration of solubilised product • High energy demand
Dilute acid	• High reaction rate	• Presence of fermentation inhibitors • Acidic condition • High construction cost • Require neutralization process
Alkali	• Low reagent cost • Require less safety requirement	• Low effectiveness of lignin removal • Require huge amount of water in washing step • Presence of irrecoverable salt
Microbial	• Low capital cost • Low energy • No addition of chemical • Mild environment conditions	• Control of growth conditions • Require large space • Slow rate for industrial purposes • Loss of sugar
Enzymatic	• Short incubation time • Similar degradation rate with fungal pretreatment • Low loss of sugar • Low inhibitory or toxic compounds	• Higher costs compared to microbial pretreatment

2.4 Lignin

Ligninocellulosic biomass is mainly made up from three components: lignin (6% - 32%), hemicellulose (7% - 38%) and cellulose (20% - 65%) (Limayem and Ricke, 2012, Goh et al., 2010). Lignin is an aromatic compounds with a molecular weight of 10 kDa (Limayem and Ricke, 2012). The lignin is formed through the oxidative

polymerisation from three hydroxycinnamyl alcohol monomers or monolignols namely, coumaryl, coniferyl and sinapyl alcohol. These three monomers are the precursors of the phenylpropanoid lignin units p-hydroxyphenyl (H), guaiacyl (G) syringyl (S) and these lignin units are linked together with a variety of the ether and carbon-carbon linkages (Fig. 2.2) (Zhu et al., 2017, Vasco-Correa et al., 2016). The resultant composition of and structure of lignin in lignocellulosic biomass is dependent on the relative concentration of each monolignols. After the polymerisation of the monolignols, lignin is cross-linking with hemicellulose and cellulose through covalent and hydrogen bonding to form a complex matrix. The complexity and rigidity of this matrix protects the plant from microbial and pathogen (Tuomela et al., 2000, Bugg et al., 2011a, Wang et al., 2013b) but it also restricts the accessibility of hydrolytic enzymes to cellulose in the hydrolysis process and reduce the liberation of fermentable sugars, which are the substrate for the bioethanol production (Vasco-Correa et al., 2016). Thus, lignin must be degraded or removed to increase the liberation of fermentable sugars. Extensive studies showed that lignin can be degraded by ligninolytic enzymes (Feng et al., 2011, Singhal and Rathore, 2001, Zhang et al., 2020).

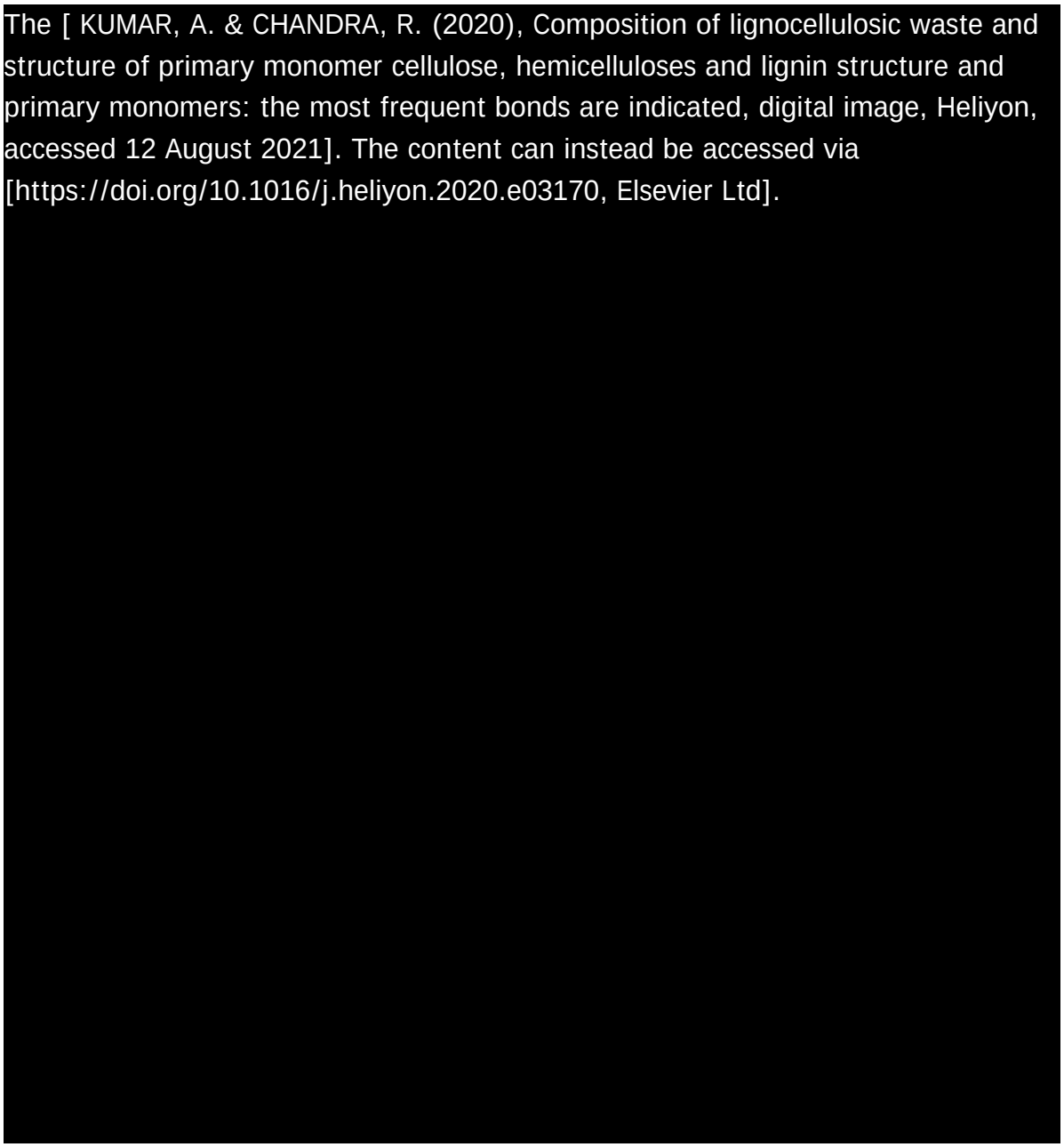


Figure 2.2 Lignin structure and its intermolecular bond; lignin primary monomers (Kumar and Chandra, 2020)

Figure 2.3 is the illustration on current model for lignin degradation proposed by Brown and Chang (2014). The lignin degradation involve generation of the radical mediators by metalloenzymes such as veratryl alcohol cation radical and various Mn(III) coordination complexes. These mediators diffuse to the lignin substrate to generate radical sites within the lignin. Upon formation of a lignin-based radical, bond scission reactions will take place and lead to its depolymerization to smaller aromatic compounds, CO_2 and water. Given the complexity of the lignin structure, the identity and properties the full enzyme and mediator systems that are involved in the lignin metabolism have yet to be elucidated. To date, β-aryl ether degradation, biphenyl

degradation pathway and diaryl propane degradation pathway are the common lignin degradation pathway by bacteria on certain lignin component that have been identified (Bugg et al., 2011b).

The [BROWN, M. E. & CHANG, M. C. Y. (2014), Microbial degradation of lignin. (b) The current model for lignin degradation involves enzymatic generation of the radical mediator, which can then diffuse to the lignin substrate and transfer the oxidizing equivalent to the polymer. Upon formation of a lignin-based radical, bond scission reactions will ensue that lead to depolymerization, digital image, Current Opinion in Chemical Biology, accessed 15 September 2020]. The content can instead be accessed via [http://dx.doi.org/10.1016/j.cbpa.2013.11.015, Elsevier Ltd].

Figure 2.3 Proposed model for microbial degradation of lignin involves enzymatic generation of radical mediator/oxidant, which can diffuse to the lignin structure and react to form radical site within the lignin and initiate a bond scission reactions that lead to depolymerisation (Brown and Chang, 2014)

2.5 Lignin Degrading Microorganisms and Enzymes

The main ligninolytic enzymes are comprised of Lignin Peroxidase (LiP, EC 1.11.1.14), Manganese Peroxidase (MnP, EC 1.11.1.13) and Laccase (Lac, EC 1.10.3.2) and they act in concertedly in breaking down the lignin. Ligninolytic enzymes could be obtained from microorganisms, such as fungi, bacteria and actinomycetes. Study by DeAngelis et al (2011) and Anukam et al. (2020) revealed that microbial communities of forest soils and termite gut may actively contribute to the decomposition of lignin (Anukam et al., 2020). Thus, most of the lignin degrading microorganisms were isolated from soils sample, compost, agricultural wastes (wheat straw, rice straw, bagasse) and industry waste (pulp and paper effluent) as these are the natural habitat of ligninolytic microbes.

2.5.1 Lignin Degrading Fungi

White Rot Fungi (WRF) are the most studied group of lignin degrading microorganisms, due to its capability in producing a range of strong oxidative and low substrate specificity extracellular ligninolytic enzymes (LiP, MnP and Lac). WRF can degrade lignin to CO_2 whereas brown rot fungi only can modify the lignin structure. Among the WRF, *Phanerochaete chrysosporium* is the most studied lignin degrader due to its ability in producing diverse enzymes (Lac, MnP, LiP) (Kirk et al., 1986,

Paszczyński et al., 1988, Dittmer et al., 1997) and high lignin degradability. Several studies showed that *P. chrysosporium* is capable in degrading various lignocellulosic biomass such as EFB, rice straw and eucalyptus globulus wood (Hamisan et al., 2009, Piñeros-Castro and Velásquez-Lozano, 2014, Chang et al., 2012, del Río et al., 2001). It has been reported that *P. chrysosporium* could degrade 41% of empty fruit bunch, 28.3% of rice straw and 15% of eucalyptus (Piñeros-Castro and Velásquez-Lozano, 2014, Chang et al., 2012, del Río et al., 2001). Chang et al. (2012) suggested that the lignin degradation by *P. chrysosporium* could be attributed to the actions of LiP and MnP since laccase was not detected in both Solid-State Fermentation (SSF) and Submerged Fermentation (SmF) of rice straw. Apart from producing LiP and MnP, several *P. chrysosporium* strains also have been reported to be capable in producing laccase enzyme (Jhadav et al., 2009, Kwak et al., 2017, Sharma et al., 2017). Due to its versatility, *P. chrysosporium* is often used in lignin degradation and other bioprocesses (Piñeros-Castro and Velásquez-Lozano, 2014, Singh and Chen, 2008). To increase the enzyme production by *P. chrysosporium*, quite a few research studies relevant to optimisation of the culture conditions have been reported. Studies showed that varying the nutritional condition and fermentation configuration and addition of inducers (redox-mediator, surfactants and etc.) could enhance the enzyme production (Govumoni et al., 2015, Dittmer et al., 1997, Wang et al., 2008, Sharma et al., 2017, Kheiralla et al., 2013, Leěstan et al., 1993). In recent year, genetic engineering has gained substantial interest in modifying the genetic of the wild type *P. chrysosporium* to increase its enzyme production and delignification performance (Coconi-Linares et al., 2014, Coconi Linares et al., 2018). Coconi-Linares et al. (2014) has sucessfully co-expressing manganese peroxidase (*mnp1*) and lignin peroxidase (*lipH8*) from wild type strain *P. chrysosporium*, and the codon-optimised Versatile Peroxidase (*vpl2*) gene from *Pleurotus eryngii*. Generally, a significant increment in the enzyme activity was observed in the recombinant strains. This result is in accordance with the recent studies on overexpressing laccase and peroxidase in *P. chrysosporium* by the same group of researchers. Moreover, it was found out the recombinant fungus *P. chrysosporium* has improved about 25% of lignin degradation in wheat bran and sugarcanoptimale bagasse when compared to the wild type fungus.

Apart from the traditional fungus *P. chrysosporium*, quite a number of white rot (*Pleurotus ostreatus*, *Tramates hirsuta*, *Tramates gibossa*, *Dichomytus squalens*)

(Piñeros-Castro and Velásquez-Lozano, 2014, Knežević et al., 2016, Knežević et al., 2013) and brown rot fungi (*Fusarium moniliforme*, *Fusarium concolor*, *Fusarium pinicola*) (Chang et al., 2012, Li et al., 2008, Knežević et al., 2013) were proven to be much efficient in lignin degradation process in comparing to *P. chrysosporium*. Piñeros-Castro and Velásquez-Lozano (2014) detected 50% loss of lignin from empty fruit bunch after 3 weeks of fermentation by *P. ostreatus*, while Chang et al. (2012) noted a loss of 34.7% after 10 day cultivation in rice straw by *F. moniliforme*. Moreover, these strains has high degrading selectivity towards lignin while maintaining low degradation in cellulose and similar phenomena was also observed in *Fusarium concolor*, whereby only 7.62% of holocellulose was lost after 5 days of fermentation in wheat straw (Li et al., 2008). Cellulose is the feedstock for the subsequent fermentation into bioethanol, thus, isolate that is able to degrade lignin selectively while maintaining the cellulose content hold better prospects in various biotechnological applications than the traditional fungus.

Fungi produce lignin degrading enzymes in different combinations and the enzymes work synergically in degrading the lignin. There are several combinations including MnP-Lac, LiP-MnP, laccase-LiP, LiP-MnP-laccase and laccase only (Hatakka, 1994, Vares et al., 1995). The level of enzyme production, combination of ligninolytic enzymes and lignin degradability of the fungi are highly dependent on the strain and culture condition. Among the ligninolytic enzymes, families of laccase obtained from fungi have been extensively studied. Several studies showed that laccase was the primary ligninolytic enzyme produced by fungus such as *Trametes hirsuta*, *Pleurotus ostsreatus*, *Fusarium concolor* (Knežević et al., 2016, Dwivedi et al., 2010, Li et al., 2008). Laccase producing fungi are widely used in textile industrial and wastewater in removing dye and phenol content. In recent study, Zerva et al. (2017) reported that two laccase producing wood-rot fungi were capable in removing more than 90% of the phenols in the olive mill wastewater within 24 days (Zerva et al., 2017). Similarly, Jönsson et al. (1998) also demonstrated that the WRF *Trametes versicolor* was able to remove 94% of phenolic content in lignocellulose hydrolysate by using laccase enzymes (Jönsson et al., 1998).

As for dye removal application, laccase producing fungi such as *C. clodosporioides*, *Lentinus polychrous* Lev. and *Leptosphaerulina* sp. (Da Silva et al., 2008, Sarnthima et al., 2009, Copete et al., 2015) were found to be effective in

removing various groups of dye (azo, di-azo, indigo, triarylmethane). Sarnthima et al. (2009) have utilised different types of agro-industrial wastes (rice husk, rice bran) to enhance the laccase production, and this laccase enzyme was able to work in broad range of pH from 3.0 to 9.0 with maximum decolorisation of 90% within 3 hrs. Copete et al. (2015) also demonstrated that 92.2% of Methyl Orange, 80.7% of Evans Blue, 83% of Orange II, 91.3% Acid Blue and 56.1% Remazol Brilliant Blue were being removed by laccase obtained from *Leptosphaerulina* sp. in the presence of redox-mediators.

Numerous wood-rot fungi (*Pleurotus eryngii, Pleurotus ostreatus, Trametes versicolor, Dichmomitus squalens, Formitopsis pinicola, Lenzites betulinus*) were reported to be capable in producing ligninolytic enzymes in the combination of MnP-laccase (Knežević et al., 2013). Based upon this work in these fungi, the best MnP producers (*Dichomytus squalens and F. pinicola)* achieved relatively greater delignification up to 34.1% and 32.4% comparing to the best Lac producer (*Pleurotus eryngii* and *Pleurotus ostreatus)*. Thus, it is possible that lignin loss could be attributed mainly to the action of Mn-oxidising peroxidases since lignin degradation in wheat straw was not associated with high laccase activity. *Pleurotus eryngii* and *Pleurotus ostreatus* possessed lowest lignin degradation of 7.1% and 14.5% although it has relatively higher overall enzyme and laccase isoforms. Moreover, it was observed that level of ligninolytic enzymes did not correlate with the rate of lignin degradation in wheat straw in some cases. Similar behaviour was observed in several *Trametes* species (Knežević et al., 2016) whereby high lignin degradation was noted by low ligninolytic enzyme level *Trametes hirsuta* and *Trametes gibbosa* with lower ligninolytic enzyme level.

Reports on lignin degrading fungi that can produce LiP-MnP-laccase are limited as compared to the other combination of ligninolytic enzymes, however, there are numerous fungi that have been reported (Vares et al., 1995, Bonugli-Santos et al., 2010). LiP-MnP-laccase was detected in the cultivation of *Phlebia radiata* during SSF of wheat straw under low nitrogen liquid medium. Different species of white rot fungus *Trametes* (*T. Versicolor, T. hirsuta,* and *T. ochracea*) were reported to be producing LiP and MnP along with laccase (Tomšovský and Homolka, 2003). The simultaneous production of LiP, MnP and laccase by marine fungi (*Aspergillus sclerotiorum, Cladosporium cladosporoiodes* and *Mucor racemosus*) have also been first revealed

by Bonugli-Santos et al. (2010). These organisms were capable in producing LiP, MnP and laccase in the malt extract medium supplemented with NaCl, however, LiP was absent when these fungi were cultivated in Basal medium. Thus, it is suggested that the production of ligninolytic enzymes is highly dependent on the culture medium.

2.5.2 Lignin degrading bacteria

To date, lignin degrading fungi has been well studied as compared to bacteria. Although fungi are well-equipped with its lignin degradability, there are also evidence showing that bacteria are involved in lignin degradation process. In 1995, Thambirajah and his group studied the microbial environment and population present in the EFB composting process. Composting is a natural lignin degradation process whereby the lignin is being degraded in the early stage to obtain valuable degraded products. In the early stage of the EFB composting process, the compost temperature could easily increase to 70^{o}C which is known as thermophilic phase. Study suggested that the microbial population in composting was dominated by bacteria that exhibited greater survival rate than fungi in the thermophilic stage (Thambirajah et al., 1995). This observation was in accordance with several studies showing that total bacteria population increased tremendously as the process entered thermophilic phase and remained active in the entire thermophilic phase (Lim et al., 2009, Ahmad et al., 2011b). The bacteria succession study revealed that phylum *Firmicutes*, *delta-proteobacterium*, *proteobacteria* and *chloroflexi* and *Bacillus* sp. were the dominant bacteria species present throughout the EFB biodegradation process (Mohd Zainudin et al., 2014, Zainudin et al., 2013, Baharuddin et al., 2009). This showed that EFB is a good source in isolating thermophilic lignin degrading bacteria. Apart from that, a thermophilic rumen ligninolytic bacterium RUFR60 was documented to be beneficial for improving rice straw lignocellulosic degradation by reducing lignin content from 16.89% to 9.17% at optimal degradation period of 15 days (Seesatat et al., 2021). Similarly, a thermostable microbial composite, AC-1 that made up from *Tepidimicrobium*, *Haloplasma*, *norank-f-Limnochordaceae*, *Rumiclostridium* and *Rhodothermus* attained about 20.12% of lignin degradation efficiency in rice straw at 60℃ (Liu et al., 2021b). The findings indicated that thermophilic bacteria possessed lignin degrading ability and play an important role in lignin degradation process. Extensive studies have been done on isolation of bacteria from soils, agriculture wastes

(wheat straw, bagasse) and industrial waste (paper and pulp effluent). The bacteria were classified into different phylum and lignin degrading bacteria are commonly found in phylum proteobacteria, actinobacteria and firmicutes.

2.5.2.1 Proteobacteria

In 2014, Woo et al. (2014) had conducted a study on diversity and enzyme activities of lignin degrading bacteria isolated from wet tropical forest soils. The finding revealed proteobacteria is the most populated phylum for their lignin degrading ability and ligninolytic enzyme production. These can be further classified into γ-proteobacteria, α-proteobacteria and β-proteobacteria (Woo et al., 2014). Natural lignin is a very complex structure due to the variety of monolignols and the irregular linkages among these monolignols (Yang et al., 2008). Thus, most of the lignin degradation studies were performed using lignin derivatives (phenolic compounds, dyes), lignin dimer model and very few with natural lignin. Apart from that, kraft lignin and black liquor lignin are the common lignin derivatives compound that had been examined in the previous degradation study (Chandra and Abhishek, 2010, Chandra et al., 2011, Chandra and Bharagava, 2013, Shi et al., 2013b, Zheng et al., 2014). Table 2.3 summarises the recent study on lignin degradation by proteobacteria. In the class of γ-proteobacteria, numerous lignin degrading strains were found in the genera belonging to *Pseudomonas*, *Citrobacter* and *Acinetobacter*.

Table 2.3 Previous studies on isolation of ligninolytic proteobacteria

Microorganisms	Enzymes	Sources	Lignin degrading capacity	Reference
γ-proteobacteria				
Acinetobacter jumii	ND[1]	Textile plant wastewater	98% Reactive-red 120 & 55.1% Cr(VI)	(Anwar et al., 2014)
***Pantoea* sp. Sd-1**	ND[1]	Rice seeds	54.5 % pretreated rice straw; 69.1% Kraft lignin	(Xiong et al., 2014)
β-proteobacteria				
***Burkholderia* sp.**	Peroxidase	Wet tropical forest soils	ND[1]	(Woo et al., 2014)
***Comamonas* sp. B-9**	Lac, MnP	Bamboo slips	Able to degrade vanillin, vanillic acid and 4-hydroxy-3-methoxy benzenepropanol	(Niladevi and Prema, 2005)
***Comamonas* sp. B-9**	Lac, MnP	Bamboo slips	43.5 % of lignin from black liquor	(Raj et al., 2014)
***Cupriavidus basilensis* B-8**	Lac, MnP	Bamboo slips	44.4% of Kraft lignin	(Shi et al., 2013a)
***Pandoraea* sp. B-6**	Lac, MnP	Bamboo slips	46.5% of Kraft lignin	(Ravindran and Jaiswal, 2016)
***Pandoraea* sp. ISTBK**	Lac, MnP, LiP	Soil sample	36.2% of Kraft lignin	(Kumar et al., 2015)
α-proteobacteria				
***Novosphingobium* sp.**	Peroxidase	Wet tropical forest soils	ND[1]	(Woo et al., 2014)
***Novosphingobium* sp. B-7**	Lac, MnP	Bamboo slips	34.7% Kraft lignin	(Chen et al., 2012a)
***Rhizobiales* bacterium**	ND[1]	Woodland sites	ND[1]	(Taylor et al., 2012)

ND[1]: Not determine.

Among the ligninolytic γ-proteobacteria, *Pseudomonas* strains were first found to exhibit significant lignin degrading activity. The isolated *Pseudomanas* sp. from a lake by Haider et al. (1978) showed significant release of $^{14}CO_2$ during degradation of ^{14}C-labelled dehydrapolymer of coniferyl alcohol (DHP) or corn stalk lignin. A number of *Pseudomonas* strains were also capable in cleaving aromatic compounds (p-hydroxybenzoic, vanillic, veratric or anisic acids) through o-cleavage (Kumar et al., 2001). *Pseudomonas* sp. isolated from paper and pulp effluent was also able to degrade lignin content in pulp and paper effluent. The degree of lignin reduction was determined by measuring the chemical oxygen demand (COD) in the effluent since the level of COD is directly correlated with the lignin content. It was reported that 60.3% COD, 70.7% biochemical oxygen demand (BOD) and 20.3% colour reduction was observed in 72 hrs of incubation at 35℃ (Vadakenat and Babu, 2014). A laccase producing *Pseudomonas putida* strain has demonstrated its decolorisation ability in several synthetic dyes (bromophenol blue, brilliant green, crystal violet, congo red) and paper effluent. Comparative study has been made in the treatment of synthetic dyes and paper effluent with culture supernatant and culture of P. *putida*. In general, greater performance was observed in culture of *P*. *putida* treatment by achieving 58-93% decolorisation of synthetic dye and effluent whereby culture supernatant only obtained 16-94% of decolourisation. Similar decolorisation activity was reported for the *Pseudomonas* ETL-1942 strain, which can rapidly degrade various synthetic dyes such as Remazol Orange H2R, Remazol Orange 3R and Remazol Black B within 24 h (Shah et al., 2013). Apart from decolorisation application, *Pseudomonas chlororaphis* PT02 strain showed the ability in mineralising aromatic compounds (syringaldehyde, guaiacol, veratryl alcohol) as the sole carbons in minimum medium (Tian et al., 2016). Metabolism of aromatic compounds by *Pseudomonas* strain indicates its potential usage in lignin degradation process. *Stenotrophomonas maltophilia* PT03 also can utilise some dye and aromatic compounds as sole carbon source. The ability in degrading the dye indicated the presence of oxidative enzyme in the culture of *S. maltophilia* PT03 in both Luria Broth medium and Minimum salt medium (Tian et al., 2016). *Pontoea* sp. Sd-1, a rice endophytic bacterium has also been reported to be capable in degrading lignin content in kraft lignin and rice straw (Xiong et al., 2014).

Several strains from *Citrobacter*, another genus of γ-proteobacteria also showed ligninolytic activity. Two *Citrobacter* strains (*Citrobacter* sp. and *C. freundii*) were isolated from paper and pulp effluent. In addition, mixed culture of *Citrobacter* sp. and *C. freundii* showed greater lignin degradability in black liquor comparing to their axenic culture. Interestingly, mixed culture of *Citrobacter* sp. and *C. freundii* were able to degrade lignin while utilising its constituents (2,4,6-trichlorophenol, pentachlorophenol) that has inhibitory effect in axenic culture as sole carbon, nitrogen source and energy sources (Chandra and Abhishek, 2010). MnP enzymes was found to be present in both axenic and mixed culture, thus, it was suggested that the lignin degradation was attributed to MnP enzymes. Apart from MnP enzymes, a dimeric nature LiP enzyme (55-65 kDa), another primary enzyme was found in *Acinetobacter calcoaceticus*. This LiP enzyme was produced by *Acinetobacter calcoaceticus* internally and it has a low substrate specificity whereby it could oxidise variety of high and low redox potential substrates namely hydroquinone, *n*-propanol, veratryl alcohol and different group of dyes (azo, heteocyclic, thiazin and polymerics group) (Ghodake et al., 2009). The decolorisation ability was also observed in *Acinetobacter junii* FA10, which showed 94.8% of colour reduction in Reactive Red-120. In addition, it is noteworthy that this strain has high tolerance towards metal and salt. Significant decolourisation was noted even at salinity as high as 150 $g\ L^{-1}$. Moreover, it was reported that *Acinetobacter junii* FA10 has the unique ability in removing chromium (82%) and Reactive Red-120 dye (98%) simultaneously (Anwar et al., 2014). The above mentioned γ-proteobacteria strains were mainly aerobic bacteria and lignin degradation was carried out in the presence of oxygen. However, DeAngelis et al. (2013) have detected significant lignin reduction in a facultative anaerobe, *Enterobacter lignolyticus* SCF1. DeAngelis et al. have used transcriptomics and proteomic to monitor the growth of *Enterobacter lignolyticus* SCF1 grown on xylose minimal media with and without lignin. It was found that the growth was enhanced, and the proteins (catalase/ peroxidase, DypB-type peroxidase) involved in lignin degradation were up-regulated when SCF1 was cultured in xylose minimal media with addition of lignin as extra carbon source. It is noteworthy that no inhibitory effect was observed on the growth of SCF1 by the soluble lignin degradation products, mainly low molecular weight of aromatic compounds. Thus, this unique property makes *Enterobacter lignolyticus* SCF1 a potential lignin degrader (DeAngelis et al., 2013). Two *α-Proteobacteria* strains from *Serratia* genus (*Serratia* sp. JHT01 and *Serratia*

liquefacien PT01) were also shown to utilise dye as sole carbon souces and significant decolorisation was observed in Methylene Blue (MB) and Remazol Brilliant Blue R (RBBR). In addition, these two strains were also able to mineralise lignin derivatives such as guaiacol, veratryl alcohol and biphenyl compounds (Tian et al., 2016). Recently, isolation of potential lignin degrading bacteria was extended to thermophilic bacteria. A novel thermophilic and an alkali-tolerant strain Serratia sp. AXJ-M exhibited lignin activities was successfully isolated from soil in pulp and paper mill. The black liquor from papermaking effluent containing 1500 mg/L lignin with 19,300 mg/L COD was treated with *Serratia* sp. AXJ-M at 50℃ for 7 days. This strain showed 60% of lignin reduction in black liquor bioremediation treatment using *Serratia* sp. AXJ-M.

Lignin degradation was also reported in some *β-Proteobacteria* isolated from bamboo slips and they were capable in producing oxidative enzymes in the combination of MnP-laccase enzyme, but no LiP was detected (Shi et al., 2013a, Shi et al., 2013b). A MnP-Lac enzyme producer, *Cupriavidus basilensis* B-8 has showed kraft lignin degrading capacity as high as 44.4%. It was suggested that MnP was responsible in the entire lignin degradation process, whereas Lac mainly active in the latter stage since its maximum production was on day 4 of incubation. Metabolic products such as guaiacol related compounds, cinnamic acid and p-hydroxyphenyl units were detected in the culture of *Cupriavidus basilensis* B-8. The metabolites mentioned above are basic monomers for guaiacyl units and p-hydroxyphenyl units. Study showed that these metabolites were produced through three catabolic pathways such as the β-ketoadipate central pathway, methylcatechol *ortho* ring-cleavage pathway and the gentisate pathway (Shi et al., 2013a). Phenol oxidase and peroxidase activity were also found in *Cupriavidus* sp., which was isolated from wet tropical forest soils (Woo et al., 2014). *Comamonas* sp. B-9 also have been reported in involving kraft lignin degradation by using MnP-Lac enzyme and low molecular weight compounds were found in the lignin degradation (Chen et al., 2012b). Similar behaviour has been found in strain *Pandoraea* sp. B-6 whereby its highest lignin degrading capacity was observed at pH 10 (Shi et al., 2013b). In 2015, another alkalotolerant strain, *Pandoraea* sp. ISTKB was reported by Kumar et al. (2015). Three primary enzymes (LiP, MnP , Lac) were found to be present in culture of *Pandoraea* sp. ISTKB (Kumar et al., 2015). The unique ability of these two strains

working in alkaline condition is greatly beneficial in industrial lignin utilisation. In contrary, another strain *Pandoraea norimbergenesis* LD001 showed a rather limited utilisation of lignin-associated monomers although this strain showed good growth in both high and low-molecular weight lignin. Numerous rare and novel lignin degraders were also found in other *β*-proteobacteria from genera belonging to *Aquitalea, Gulbenkiania* and *Pseudogulbenkiania*, *Variovorax* and *Roseateles* (Woo et al., 2014). Phenol oxidase and peroxidase oxidative enzymes were found to be present in these genera.

Metabolism of lignin-associated compounds was observed in the *α-Proteobacteria* class. The genus *Novosphingobium* contains aerobic strains including *Novosphingobium* sp. and *Novosphingobium* B-7, which were isolated from wet tropical forest soils and steeping fluid of the eroded bamboo slips (Woo et al., 2014). Phenol oxidase and peroxidase activity was found in *Novosphingobium* sp. but no further lignin degradation study was carried out. On the contrary, different enzymes (MnP-Lac) were produced by *Novosphingobium* B-7 to degrade lignin under minimal salt medium with kraft lignin as the sole carbon. The presence of phenolic units and low molecular weight alcohols has clearly indicated the capability of *Novosphingobium* B-7 in depolymerising lignin structure since these compounds are considered as the monomers of the natural lignin polymer (Chen et al., 2012a). Ligninolytic enzyme activity was also observed in genera of *Azospirillum* and *Ochrobactrum*. Thermo tolerant bacterial laccase was first found in the rhizospheric *α-Proteobacterium Azospirillum lipoferum* with high thermostability even at 70℃ (Diamantidis et al., 2000) however, no lignin degradation study was done. In 2015, several *Azospirillum* strains from *A. brasilense, A. picis, A. lipoferum* and *A. tiophilum* species were revealed to be able to produce phenol oxidase, MnP and LiP. These strains were capable of decomposing lignin model compounds, both native and methanolysed sawdust. Generally, most of the studied strains showed greater lignin degradability in methanolysed sawdust since it has less compact lignin structure and contain more phenol derivatives. In contrast, endophytic *A. brasilense* Sp245 was more efficient in degrading native sawdust. It was suggested that this could be due to the symbiotic effect between endophytic bacteria with the native sawdust. Therefore, endophytic bacteria were known to possess greater lignin-degrading potential

compared to associative strains due to their penetration capabilities (Kupryashina et al., 2015).

Several potential lignin degraders were also revealed in genus *Rhizobium.* *Rhizobium petrolearium* strain SL-1 shown to metabolise polycyclic aromatic hydrocarbons (Zhang et al., 2012). Similar activity was observed in *Rhizobium* sp. strain YS-r1 where this strain was capable of degrading even greater variety of substrates including lignin-like compounds (*p*-anisoin, vanillic, ferulic acid) and native lignin such as switchgrass and alfafa. Gene encoding for classical ligninolytic enzymes (LiP, MnP and VP) were not observed in strain YS-r1. However, this strain exhibited LiP-like enzymes and the genome analysis revealed the presence of variety gene encoding for lignin-oxidising and H_2O_2-producing enzymes. Thus, it was inferred that the degradation in the native lignin by *Rhizobium* sp. strain YS-r1 was due to the synergy effect between LiP-like enzymes, various lignin-oxidising and H_2O_2-producing enzymes (Jackson et al., 2017). Apart from lignin degradation application, *R. radiobacter* showed its potential in dye removal application by achieving 80-95% of decolorisation efficiency in various azo, triphenylmethane (TPM), disperse and reactive textiles (Telke et al., 2008).

2.5.2.2 Firmicutes

As shown in Table 2.4, most of previous lignin degradation studies on phylum *Firmicutes* were found in Bacilli class. Various *Bacillus* strains were found to produce LiP, MnP and Lac enzymes extracellularly (Naz et al., 2015, Abdul Rahman et al., 2013). MnP was the dominant enzyme in strain *Bacillus* sp. SHC 1, while *Bacillus* sp. which was isolated from agro field prove the contrary whereby LiP was the dominant enzyme instead of MnP. Apart from extracellular enzyme, intracellular LiP was secreted by *Bacillus* sp. strain which was isolated from sediment core of pulp and paper mill industries (Kharayat and Thakur, 2012). Another *Bacillus* sp. strain isolated from Egyptian soil was able to utilise commercial kraft lignin as sole carbon and showed 81.4% of lignin degradation (Abd-Elsalam and El-Hanafy, 2009). Similarly, two *Bacillus* sp. strains, isolated from pulp paper waste and sludge of pulp and paper mill also demonstrated 30% and 37% of kraft lignin respectively (Chandra et al., 2007,

Chandra and Bharagava, 2013). The degradation was further confirmed by the presence of low molecular weight aromatic compounds such as t-cinnamic acid, 3,4,5-trimethoxy benzaldehyde, ferulic acid during degradation process (Chandra et al., 2007). *Bacillus subtilis* CS-1 also showed its degradability on native lignin by degrading 20% of Klason lignin and 19.2% of hemicellulose in rice straw (Chang et al., 2014). Capability of degrading lignin model dimer guaiacylglycerol-β-guaiacyl ether was also observed in *Bacillus pumilus*, *Bacillus atropheus* strain (Huang et al., 2013). *Bacillus cereus* have proven capacity to degrade another lignin related compound namely pentachlorophenol and phenol (Singh et al., 2009). A thermostable laccase gene (*cotA*) from *B. licheniformis* also demonstrated its capability in catalysing dimerization of phenolic acids such as sinapic, ferulic and syringic acids (Koschorreck et al., 2008).

Table 2.4 Previous studies on isolation of lignin degrading bacteria from phylum Firmicutes

Microorganisms	Enzymes	Sources	Lignin degrading capacity	Reference
***Acetoanaerobium* sp. WJDL-Y2**	ND[1]	Pulp and paper mill effluent	24.9 % of Kraft lignin	(Duan et al., 2016)
Aneurinibacillus aneurinilyticus	ND[1]	Sludge of pulp and paper mill	33% of Kraft lignin	(Chandra et al., 2007)
Aneurinibacillus aneurinilyticus	ND[1]	Sludge of pulp and paper mill	43% of Kraft lignin	(Raj et al., 2006)
***Bacillus amyloliquefaciens* SL-7**	LiP, MnP, Lac	Tobacco straw	55% of straw lignin	(Mei et al., 2020)
Bacillus atrophaeus	Lac	Rainforest soils	27.5% of Guaiacylglycerol-β-guaiacyl ether (GGE)	(Huang et al., 2013)
Bacillus cereus	ND[1]	Pulp paper mill effluent sludge	91.63%	(Singh et al., 2009)
Bacillus pumilus	Lac	Rainforest soils	35.1% of Guaiacylglycerol-β-guaiacyl ether (GGE)	(Huang et al., 2013)
Bacillus sp.	Lac, MnP, LiP	Agro-field	ND[1]	(Naz et al., 2015)
***Bacillus* sp.**	ND[1]	Sludge of pulp and paper mill	37 % of Kraft lignin	(Raj et al., 2007)
***Bacillus* sp. B37**	Lac	Soils	61.0 % Alkali lignin	(Chang et al., 2014)
Paenibacillus glucanolyticus	ND[1]	Pulping waste black liquor	ND[1]	(Mathews et al., 2014)
***Paenibacillus* sp.**	ND[1]	Pulp paper mill effluent sludge	30% Kraft lignin	(Chandra et al., 2007)

***Paenibacillus* sp.**	**ND[1]**	**Pulp paper mill effluent sludge**	**53.86% of phenol**	**(Singh et al., 2009)**
***Paenibacillus* sp.**	Lac	Pulp paper effluent	54.0% Lignin	(Anwar et al., 2014)
Ureibacillus terrenus	ND[1]	Empty Fruit Bunch	ND[1]	(Ting et al., 2013)

ND[1]: Not determine

Bacillus was selected as a promising source of ligninolytic enzyme due to its wide application not only in lignin degradation, but also in dye decolorisation. *Bacillus* sp. isolated from decomposing wood logs' soil exhibited extensive dye-decolorising capacity in recalcitrant phenothiazine dye class (Azure B, Methylene Blue and Toluidene Blue O) (Bandounas et al., 2011). Numerous studies have revealed the dye decolorisation ability of *Bacillus* was mainly attributed to laccase enzyme. A thermostable laccase produced by mesophilic strain *Bacillus amyloliquefaciens* was capable of decolourising Reactive Blue dye at elevated temperature up until 80℃. This observation is not documented for fungal laccases (Lončar et al., 2013). Laccase from two mesophiles, *B. licheniformis* LS04 and *B. pumilus* W3 also showed great stability at high temperature whereby 16% and 45% of initial laccase activity was found to be retained after incubating at 80℃ for 10 h respectively (Lu et al., 2012, Guan et al., 2014) . The most remarkable property exhibited by the laccase obtained from *B. licheniformis* LS04 and *B. pumilus* W3 was its tolerance toward alkaline and organic solvent. This mediated laccase exhibited greater decolorising efficiency on azo and anthraquinonic dye under alkaline condition. These unusual properties indicated a high potential of bacterial laccase in industrial application.

Paenibacillus, another genus of the *Bacilli* class also contains strains capable of degrading lignin derivatives compounds. Phenol removal ability was revealed in several *Paenibacillus* sp. strains. A laccase producing *Paenibacillus* sp. strain LD-1 has effectively detoxify pulp and paper mill effluent by reducing 86% of phenolic compounds including 2-methoxyphenol, 2,6-dimethoxy phenol and benzoic acid (Raj

et al., 2014). Another *Paenibacillus* sp. which was isolated from pulp paper mill effluent also showed 53.85% phenol removal (Singh et al., 2009). However, the degradation only occurs in the presence of glucose. Thus, it is believed that this *Paenibacillus* strain was glucose dependent, and it could only utilise phenol compound in the presence of glucose which indicated the phenomenon of co-metabolism. Similar co-metabolism behaviour was also observed in a facultative anaerobe, *P. glucanolyticus* under aerobic condition in black liquor. Black liquor may contain a variety of carbon sources including lignin, cellulose, hemicellulose and polysaccharide degradation products such as glucose and xylose. A biphasic growth phenomenon was noted in *P. glucanolyticus* culture and this has indicated that it also can co-metabolised more than one carbon sources. Interestingly, significant lignin degradation was observed in *P. glucanolyticus* under anaerobic growth in black liquor. The presence of numerous lignin degradation products (gallic, acetic and propanoic acid) has further indicated the degradation of lignin present in black liquor (Mathews et al., 2014). Similar degradation products were also reported in lignin degradation by strain *Aneurinibacillus aneurinilyticus* which was isolated from pulp and paper mill's sludge (Raj et al., 2006). Lignin degradation was also revealed in another anaerobic strains, *Acetoanaerobium* sp. WJDL-Y2 (Duan et al., 2016) and this strain was capable to utilise kraft lignin as sole carbon source which showed a different metabolism from *P. glucanolyticus*.

2.5.2.3 Actinobacteria

Actinobacteria is another bacteria species that has been explored by researchers in recent years for its ligninolytic activity. Generally, actinobacteria are known as fungi-like bacteria. Table 2.5 summarises the previous studies on discovery of ligninolytic enzymes and lignin degradation of actinobacteria. To date, most of the lignin degrader actinobacteria were discovered in genus *Streptomyces* including *Streptomyces psammoticus, S. badius, S. cyaneus, S. violaceoruber* and *S. griseorubens* JSD-1 (Niladevi and Prema, 2008, Arias et al., 2003, Lu et al., 2014, Feng et al., 2015). Degradation of lignin-related compounds and dye decolorisation was observed in the *Streptomyces* strains mentioned above. Decolorisation of Poly R dye is often a quick method in identifying the presence of ligninolytic activity. The capability of these *Streptomyces* strains in degrading Poly R dye have indicated the presence of ligninolytic activity. Among these strain, *S. psammoticus* seem to be a

more promising lignin degrader since Niladevi and Prema (2005) reported that *S. psammoticus* NJP 49 can produce three major ligninolytic enzymes (LiP, MnP, Lac) under submerged fermentation (Niladevi and Prema, 2005).

Table 2.5 Previous studies on isolation of ligninolytic Actinobacteria

Microorganisms	Enzymes	Sources	Lignin degrading capacity	Reference
Microbacterium	ND[1]	Woodland sites	ND[1]	(Taylor et al., 2012)
***Mycobacterium* sp.**	Peroxidase	Wet tropical forest soils	ND[1]	(Woo et al., 2014)
***Nocardiopsis* sp.**	ND[1]	Empty Fruit Bunch	ND[1]	(Ting et al., 2014)
***S. cyaneus* CECT 3335**	Lac	ND[1]	18.4% kappa lignin (eucalyptus)	(Arias et al., 2003)
***S. psammoticus* NJP 49**	LiP, MnP, Lac	Marine and Mangrove	ND[1]	(Niladevi and Prema, 2005)
S. pseudogriseolus	LiP-Lac	Tropical estuarine ecosystem	99% kraft lignin degradation	(Buraimoh et al., 2015)

ND[1]: Not determine

However, not all strains from genus *Streptomyces* could produce all primary ligninolytic enzymes. For instance, only laccase enzyme was observed in strain *S. psammoticus* MTCC 7334 under both submerged fermentation and solid-state fermentation. In addition, alteration on rice straw's cell wall structure has further confirmed the occurrence of lignin degradation under solid-state fermentation with *S. psammoticus* and the degradation was mainly attributed to laccase enzymes (Niladevi et al., 2007). *S. cyaneus* CECT 3335 were also reported in producing laccase enzymes and showed considerable reduction in the kappa number of eucalyptus kraft pulps after laccase or laccase-mediator treatment (Arias et al., 2003). Interestingly, alkaline laccase with great thermal stability was found in a mesophilic strain, *S. sviceus* whereby retained about 80% residual activity after 5 days of incubation at pH 11. Besides from laccase, laccase-like multicopper oxidase (LMCO) was another ligninolytic enzyme that was potentially involved in lignocellulose degradation. Lu et al. (2014) have revealed that the sequence of LMCO obtained from *Streptomyces* spp. has high similarity to those mainly clustered with *S. violaceusniger*, *S. coelicolor* and

S. griseus (Lu et al., 2014). Similar LMCO was also produced by another *S. griseorubens* JSD-1 which was isolated from composted treated soil. This strain has the ability in utilising wheat straw as its sole carbon sources for growth (Feng et al., 2015).

Apart from laccase, several strains from genus *Streptomyces* also produce ligninolytic enzymes in the combination of LiP-Lac or peroxidase (Musengi et al., 2014). *Streptomyces* sp., *S. cinnamomensis* and *S. albogriseolus* KFG77548 (Buraimoh et al., 2015, Jing and Wang, 2012) were found to be a potent producer for LiP-Lac enzyme. Buraimoh et al. have demonstrated high lignin degradation in five indigenous *Streptomyces* strain (*S. pseudogriseolus, S. albus, S. coelicolor, S. aureus, S. albogriseolus*) that were isolated from a tropical lagoon system. Among these strains, *S. pseudogriseolus* showed the highest lignin degradation of about 99% and fastest growth with doubling time of 8.3 h. Kraft lignin degradation was occurred with the production of LiP-Lac enzyme simultaneously. Thus, it was noted that both processes support each other. Lower lignin degradation was achieved by *S. albogriseolus* KF977548 although it produced relatively greater total LiP-Lac enzyme comparing to *S. pseudogriseolus*. Apart from lignin degradation, *S. albogriseolus* KF977548 also showed complete degradation on one of the major lignin precursors (coniferyl alcohol) and several lignin aromatic metabolites (protocatechuic, vanilic, 4-hydroxybenzoic) when these substrates were used as sole carbon sources in mineral salts medium. Excretion of peroxidase ALiP-P3 enzyme was found secreted in *Streptomyces viridosporus* T7A. This LiP is capable of degrading lignin model compounds. Study by Ramachandra et al. (1988) showed that lignin treated with *Streptomyces viridosporus* T7A has successfully being degraded into CO_2 and low molecular weight compounds such as p-coumaric acid and ferulic acid (Ramachandra et al., 1988). *Streptomyces* sp. strain BSll#1 was reported to produce peroxidase that could oxidise 2,4-dichlorophenol (2,4-DCP) but this peroxidase was not classified into LiP or MnP due to limited characteristic (Musengi et al., 2014). Similar peroxidase activity was also exhibited in a thermophilic actinomycete, *S. thermoviolaceus* and this peroxidase had a half-life of 70 min at 70℃ (Asina et al., 2017).

Numerous bacteria belongings to *Rhodococcus* genus have been recognised to have the capability to degrade lignin. *Rhodococcus jostii* RHA1 received the most attention among *Rhodococcus* species. Numerous studies demonstrated that it is

capable in degrading polychlorinated biphenyl (PCB) degrader, aromatic compounds and lignin (He et al., 2017, McLeod et al., 2006, Ahmad et al., 2011a, Seto et al., 1995). High chemical value product such as vanillin was produced throughout the lignin degradation process. Ahmad et al. (2011a) demonstrated the release of vanillin was probably due to the cleavage of β-aryl ether in lignin by DyP-type peroxidase (DyP) in *R. jostii* RHA1. *R. jostii* RHA1 have gained special attention, not just because of its possible in breaking down lignin but also because of lignin to lipid conversion abilities (Hernández et al., 2008). Apart from *R. jostii* RHA1, *R. opacus* DSM 1069 and *R. opacus* PD630 also have evolved the ability to degrade lignin and accumulate lipids. Both strains could utilise vanillic acid and 4-hydroxybenzoic acid, two lignin model compounds, as carbon source and accumulate lipid under nitrogen limiting conditions (Kosa and Ragauskas, 2012). Study also showed that *R. opacus* DSM 1069 was capable in degrading complex kraft lignin structure from black liquor (Wei et al., 2015) and cofermentation of wild type *R. opacus* PD630 with engineered *R. jostii* RHA1 $VanA^-$ also showed the ability in degrading native lignin (corn stover lignin) for lipid production (He et al., 2017).

The genera *Microbacterium* and *Micrococcus* contain aerobic actinobacteria that are found in soil. The aromatic-degrading ability was revealed in *Microbacterium phyllosphaerae* and *M. marimilacus* whereby both strains were able to utilise vanillic acid and biphenyl as sole carbon source (Taylor et al., 2012). Greater aromatic degrading activity was exhibited by *Micrococcus lutues* whereby it was able to grow on veratryl alcohol, m-cresol and p-cresol that were not shown by *Microbacterium phyllosphaerae* and *M. marimilacus*. Moreover, *M. lutues* was also shown to be involved lignin degradation of palm oil mill effluents and ^{14}C-labeled rice stalks (Taylor et al., 2012, Kumar et al., 2001). Kumar et al. (2012) has demonstrated that *M. lutues* was able to use ^{14}C-labeled rice stalks as sole carbon source. Two rare actinobacteria (*Tsukamurella* sp. and *Cellulosimicrobium* sp.) relating to biodegradation of lignocellulose were isolated from agriculture soil and they were shown to be capable in producing laccase (Revollo Escudero et al., 2012). A thermo-alkali stable laccase was revealed in a rare thermophile bacterium namely *Thermobifida fusca*. This laccase exhibited high oxidation ability in several dye intermediates, especially 2,6-dimethylphenylalanine and p-aminophenol (Chen et al., 2013). Dye decolorising ability was also revealed in another novel species

Nonomuraea gerenzanensis in recent year. This strain produced a thermotolerant peroxidase that was capable in oxidise variety of dye and aromatic compounds, including ABTS, 2,6-DMP, RBBR, RB5 and AB. However, it was not able to oxidise veratryl alcohol which indicates that this peroxidase does not belong to LiP (Casciello et al., 2017).

2.6 Ligninolytic Enzymes

2.6.1 Laccase

Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) is a polyphenol oxidase that belongs to a family of multicopper enzymes. It is the most extensively studied ligninolytic enzymes. Laccase is widely distributed in fungi especially white rot fungi. *Trametes versicolor*, *Trametes hirsuta* and *Phlebia radiata* are the common fungal laccase producers (Jönsson et al., 1998, Dhakar and Pandey, 2013, Lundell and Hatakka, 1994). Laccase was not only found in fungi, but observed in some bacteria such as *Azospirillum lipoferum*, *Pseudomonas putida* and *Streptomyces* sp. (Diamantidis et al., 2000, Kuddus et al., 2013, Majumdar et al., 2014). Most of the Laccase enzymes from both fungi and bacteria are used in dye decolourisation industry with lignin degrading ability as well. Table 2.6 summarises recent studies on characterisation of laccase enzyme by fungi and bacteria.

Table 2.6 Previous studies on characterisation of Laccase enzyme from fungi and bacteria

Microorgnism	pH	Temperature (℃)	Substrate	Km (mM)	Vmax	Molecular weight (kDa)	pI	Reference
Ganoderma leucocontextum	3.0	70	Guaiacol	1.658	2.452 mM/min	65	ND	(Umar and Ahmed, 2022)
Bacillus licheniformis **NS2324 cloned and expressed in *E. coli***	8.0	40	Guaiacol	ND	ND	66	ND	(Chopra and Sondhi, 2022)
Daedalea quercine	4.5	ND	Guaiacol	0.093	ND	69	3.0	(Baldrian, 2003)
Pycnoporus cinnabariunus	ND	ND	ND	ND	ND	81	3.7	(Eggert et al., 1996)
Aspergillus nidulans	5.0	40	Guaiacol	0.052	2.86 mM/s	66	ND	(Vivekanandan et al., 2014)
Cerrena **sp.**	3	45	ABTS	0.0934	ND	58.6	ND	(Yang et al., 2014)
Cerrena maxima	3.5-4.5	50	catechol	0.122	ND	57	3.5	(Koroleva et al., 2001)
Coriolopsis brysina	3	50	ABTS	0.0316	ND	57.7	ND	(Marcinkevičienė et al., 2013)
Daedalea quercina	4	60 - 70	2,6-DMP	0.048	ND	69	3.0	(Baldrian, 2003)
Fomitella fraxinea	3	70	ABTS	0.0270	876 μM/min	47	3.8	(Park and Park, 2008)
Leptosphaerulina **sp.**	3	ND	ABTS	0.0160	ND	67.7	6.2	(Copete et al., 2015)

Magnaporthe grisea	6	30	Syringaldazine	0.118	ND	70	ND	(Dawkar et al., 2009)
Pleurotus fossulats	7	50	Guaiacol	0.083	18.37 U/min	ND	ND	(Chowdhury et al., 2014)
Pleurotus ostreatus	4	50	ABTS	0.062	570 U/mL	ND	ND	(Aslam and Asgher, 2011)
Pleurotus ostreatus	5	40	Syringaldazine	ND	ND	ND	ND	(El-Batal et al., 2015)

ND: Not determine

Laccase contains four copper sites where it consists of one type 1 Cu atom, one type 2 Cu atom and two type 3 Cu atoms. These four copper atoms are arranged in two sites, namely mononuclear (T1) and trinuclear sites (T2/T3). Each of these Cu atoms has different paramagnetic and spectroscopic properties (Abdel-Hamid et al., 2013). The catalytic mechanism of laccase is different from peroxidase enzyme where it does not require H_2O_2 to initialise the activity. It utilises O_2 as a co-substrate to generate H_2O as a non-toxic by-product. Without mediator, moderately low redox potential laccase can only oxidize phenolic compounds such as diphenols, aminophenols and aryl diamines (Wong, 2009). Laccase catalyses removal of one electron from phenolic hydroxyl groups of phenolic lignin model compounds to form phenoxy radicals. The radicals were known to spontaneously restructure that led to the cleavage of alkyl side chains of the polymer such as C_α-C_β cleavage, C_α oxidation and alkyl-aryl cleavage (Asina et al., 2017). Overall, the catalytic cycle of laccase (Figure 4) involves three major steps. The cycle starts with the transfer of electrons from substrates to the primary electron acceptor, T1 copper followed by intramolecular electron transfer from T1 copper to trinuclear copper sites (T2/T3) to produce a fully reduced enzyme. The molecular oxygen binds to T2/T3 copper site and converts reduced enzyme to a peroxide intermediate by transferring two electrons from T3 copper site. The peroxide intermediate transforms to an oxy radical by electron transfer from T1 and T2 copper, followed by $2e^-$ reductive cleavage of the O-O bond with the release of a water molecule. Lastly, a fully oxidised enzyme is formed by releasing O^{2-} as a second water molecule (Kosa and Ragauskas, 2013, Wong, 2009).

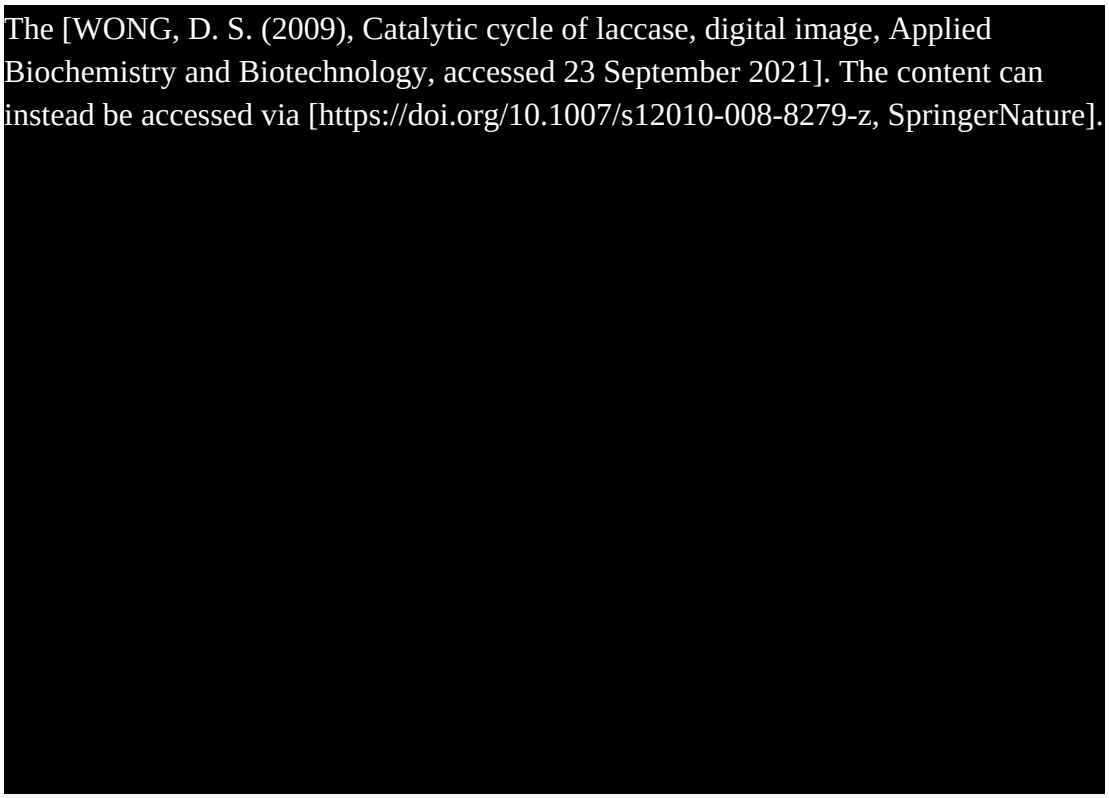


Figure 2.4 Catalytic mechanism of Laccase enzyme (Wong, 2009)

Apart from degradation of phenolic substrate, extensive studies reported that substrates for laccase can be expanded to non-phenolic compounds in the presence of mediators such as 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), 3-hydroxyanthranilic acid (HAA) and 1-hydroxybenzotriazole (HBT) (Díaz-González et al., 2011, Dwivedi et al., 2011, Morozova et al., 2007). Due to much smaller size of mediators, it serves as lignin oxidants, diffuse and attack on lignin structure via reaction of C_α-C_β cleavage, aromatic ring cleavage and β-ether cleavage. Electron transfer mechanism and hydrogen atom transfer are the common catalytic mechanism for laccase-mediator system. The reaction mechanism of laccase-mediator system is highly dependent on the type of mediators being used in the system. For mediators such as HBT, oxidation of lignin structure involves monoelectronic oxidation where mediator is oxidised by laccase to form a radical cation, followed by immediate deprotonation to form aminooxyl anion capable of abstracting a benzylic hydrogen atom from the substrate, converting it to a radical. In laccase mediator system that employs electron transfer mechanism, the mediator is oxidised to the di-cation active intermediate that is responsible for the oxidation of the non-phenolic substrate (Wong, 2009).

Substrates specificity and affinity of laccase is highly dependent on pH. Laccase demonstrate great stability at acidic condition whereby its optimal pH range from 3.0-6.0 (Park and Park, 2008, Baldrian, 2003). The pH activity profile of laccase can exhibit an optimal pH whose value depends on type of substrates. The most studied substrates for laccase enzyme are guaiacol, ABTS and 2,6 – DMP. Several studies showed that fungi laccase (*Cerrena* sp., *Coriolopsis brysina, Fomitella fraxinea* and *Pleurotus ostreatus* HP-1) exhibited maximum activity at pH 3.0 – 4.5 in both ABTS and 2,6 –DMP; and pH 4.5 – 7.0 in guaiacol. The optimal pH is still under acidic range despite the different type of substrates. Alkaline condition caused decrease in laccase activity due to binding of hydroxide ion with T2/T3 site of the laccase enzyme. Additionally, the pH affects the ionization state of the substrate, therefore affects its ability to act as reducing substrate and thus affects the enzyme activity (Patel et al., 2013). However, *Pleurotus fossulats, Streptomyces psammoticus* proved the contrary where both strains showed greater stability at alkaline condition with optimal pH 7.0 and 8.5 respectively (Chowdhury et al., 2014, Niladevi, 2008). Interestingly, laccase from *Streptomyces psammoticus* not only showed great stability at alkaline condition, also in acidic condition where 65% of residual activity was retained after incubating at pH 4 for 90 min.

Apart from pH, temperature is another factor in affecting the substrate specificity and affinity of laccase. Temperature optima of laccase activity is strain dependent. The optimal temperature for fungal laccase ranged from 30℃ to 65℃. However, several fungi including *Daedalea quercina, Steccherinum ochraceum, Marasmius quercophilus* and *Trametes versicolor* (Baldrian, 2003, Chernykh et al., 2008, Dedeyan et al., 2000, Minussi et al., 2007b) exhibited higher optimal temperature ranged between 65℃ to 80℃ . Laccase from *Steccherinum ochraneum* (1833) has showed great thermal stability where it has 1.7 hr of half-life when incubating at temperature of 70℃ . Thermotolerant laccases were also found from *Pycnoporus* genus. As compared to *S. ochraneum*, both *Pycnoporus cinnabarinus* and *P. sanguineus* (SCC 108) *strains* exhibited longer half-life at higher temperature which is 2.0 h at 80℃ and 2.8 h at 75℃ respectively (Schliephake et al., 2000, Litthauer et al., 2007). However, the fungal laccases have lower thermal stability than bacterial laccases (Petr, 2006). *Aquifex aeolicus* copper-activated metallo-oxidase, McoA was found to be thermostable with activity durable up to 9 and 5 h at 80℃ and 90℃

respectively (Fernandes et al., 2007). Similarly, the thermoactive endospore-coat laccase like enzyme of *Bacillus pumilus* W3 has retained about 45% residual activity after 10 h incubation at 80℃. The most thermostable laccase-like multicopper oxidase was found in a hyperthermophile bacteria, *Thermus thermophilus*, whereby it showed essentially long half-life of over 14 h at 80℃ (Miyazaki, 2005).

According to recent studies, the molecular weight for purified fungi laccase can vary widely from 45 – 90 kDa. The purified laccase from bacteria and actinomycetes have relatively lower molecular weight of 39.5 – 46 kDa as compared to purified fungi laccase. The pI values of purified fungi laccase ranged from 3.0 – 4.5 which is more acidic. In contrast, the purified laccase from *Leptosphaerulina* sp. and *P. sanguineus* (SCC 108) exhibited greater pI value of 6.2 and 6.7 respectively (Copete et al., 2015, Litthauer et al., 2007). Apart from this, Niladevi (2008) reported that purified laccase from actinomycetes *Streptomyces* sp. exhibited pI value of 7.9 which is slightly alkaline in comparison with purified fungi laccase.

2.6.2 Manganese Peroxidase (MnP)

MnP (EC 1.11.1.13) is known as Mn(II): hydrogen-peroxide oxidoreductase and it is classified as heme peroxidases (Wong, 2009). Mn^{3+} is a weak oxidant whereby it is only capable of oxidising phenolic compounds, which only accounts for about 10% of lignin structure. However, it has been reported that non-phenolic lignin can be oxidised by Mn^{3+} in the presence of mediator such as thiyl or lipid radicals (Abdel-Hamid et al., 2013) whereby the mediated Mn^{3+} facilitates the oxidation of non-phenolic compounds (substituted benzyl alcohol and diarylpropane structure) to their respective aldehydes. In the Mn^{3+}-thiyl mediator system, Wariishi et al (1989) proposed that thiols were oxidised to form a thiyl radical by chelated Mn^{3+} and benzylic hydrogen was subsequently abstracted from the benzyl alcohol by thiyl radical which in turn produce a benzylic radical. The benzyl radical further coupled with a thiyl radical to form an unstable thioacetal and it would later be decomposed into a benzaldehyde and a free thiol (Wariishi et al., 1989). As for Mn^{3+}-lipid radicals mediator system, a different mechanism was proposed whereby a hydrogen is abstracted from the benzylic carbon (C_α) in the non-phenolic lignin compound via lipid peroxy radicals to form a benzylic radical intermediate. Subsequently, a peroxy radical is formed by adding O_2 and followed by oxidative cleavage and nonenzymatic

degradation to produce aldehyde, which is the degradation products from non-phenolic lignin structure (Bao et al., 1994, Kapich et al., 2005, Daina et al., 2002).

Based on the summarised study in table 2.7, most of the studies revealed that the molecular weight of MnP ranged from 38 to 55.6 kDa. The range of the molecular weight does not vary as much as Laccase enzyme. In fact, the molecular weight for most of the MnP enzymes from both fungi (*Trametes versicolor*, *Irpex lacteus* and *Nematoloma frowardidd*) and bacteria (*Citrobacter* sp., *Citrobacter freundii* and *Paenibacillus* sp.) concentrated at 40 to 44 kDa. Nevertheless, Oliveira et al. (2009) showed that MnP enzymes from *Bacillus pumilus* exhibited lower molecular weight of 25 kDa. MnP is an acidic protein with pI value ranging from 3.2 to 3.9. However, de la Rubia et al. (2002) and Bermek et al. (2004) demonstrated presence of MnP enzymes with acidic and alkaline pI value of less than 2.8 and 8.2 in *Phanerochaete flavido-alba* and *Trichophyton rubrum* LSK-27 respectively (de la Rubia et al., 2002, Bermek et al., 2004).

Table 2.7 Previous studies on characterisation of MnP enzyme from fungi and bacteria

Microorganism	pH	Temperature (℃)	Substrate	Km (mM)	Vmax	Molecular weight (kDa)	pI	Reference
Cinnamomum camphora	5.0	40	2,6-DMP	0.140	220.14 U/mg	55.2	ND	(Rekik et al., 2019)
***Ganoderma lucidum* IBL-05**	5.0	40	$MnSO_4$	0.06564	640 U/ mL	43.0	ND	(Bilal et al., 2015)
Irpex lacteus	5.0	ND	$MnSO_4$	0.0467	1861.7 nkat/ mg	37.0	4.8	(Sklenar et al., 2010)
***Irpex lacteus* CD2**	4.5	70	$MnSO_4$	0.0207	ND	42.0	ND	(Qin et al., 2014)
Nematoloma frowardidd	ND	ND	ND	ND	ND	42-44	3.2	(Schneegaß et al., 1997)
Phanerochaete chrysosporium	4.5	30	2,6-DMP	0.0286	NR	45	ND	(Ürek and Pazarlioğlu, 2004)
Phanerochaete chrysosporium	ND	ND	ND	ND	ND	52.8	ND	(Koyani et al., 2013)
Phanerochaete chrysosporium	4.5	30	$MnSO_4$	0.0300	25.5 U/mg	45	ND	(Zeng et al., 2013)
Phanerochaete flavido-alba	ND	ND	$MnSO_4$	0.0519	1606 U/mg	55.6	< 2.8	(de la Rubia et al., 2002)
Phlebia radiata	ND	ND	ND	ND	ND	50	3.3-3.9	(Vares et al., 1995)

Pleurotus ostreatus	4	30	$MnSO_4$	0.033	189 U/mL	ND	ND	(Aslam and Asgher, 2011)
***Rhizoctonia* sp. SYBC-M3**	4.5	55	$MnSO_4$	0.0539	748 U/mg	40.4	ND	(Cai et al., 2010)
***Schizophyllum commune* IEL-06**	5.0	60	$MnSO_4$	0.4000	410 mM/min	40.0	ND	(Asgher et al., 2013)
***Trametes versicolor* IBL-04**	5.0	40	$MnSO_4$	0.0390	213 U/mg	43.0	ND	(Asgher et al., 2016)
***Trametes versicolor* IBL-04**	5	50	$MnSO_4$	0.070	540 U/mL	43	ND	(Asgher and Iqbal, 2011)
***Trichophyton rubrum* LSK-27**	4.5	ND	$MnSO_4$	0.063	ND	42	8.2	(Bermek et al., 2004)

ND：Not determine

2.6.3 Lignin Peroxidase (LiP)

LiP (EC 1.11.1.14) is a heme peroxidase classified in class-II peroxidase and commonly known as ligninase or diaryl propane oxygenase. LiP is commonly found in fungi and bacteria, but it is less reported in bacteria. LiP was first reported in white-rot fungus, *Phanerochaete chrysosporium* (Tien and Kirk, 1983, Glenn et al., 1983). Various LiP isozymes have been reported in *P. chrysosporium* (Leisola et al., 1987), *Trametes versicolor* (Johansson and Nyman, 1993), *Bjerkandera* sp. (Ten Have et al., 1998) *Irpex lacteus* (Rothschild et al., 2002) and *P. sordida* (Hirai et al., 2005). A potent lignin degrader, *T. versicolor* strain PRL 572 revealed about 16 isozymes of LiP designated TvLP1 to TvLP16 presence in a carbon and nitrogen-limited medium under static condition (Johansson and Nyman, 1993). Moreover, the variation and quantity of LiP isoforms in the culture medium is highly dependent on the culture condition (Reddy and D'Souza, 1994). Agitation speed and modes were proven to affect the composition of LiP isoforms in the culture of *P. sordida* and *P. chrysosporium* (Hirai et al., 2005, Podgornik et al., 2001). The study by Podgornik et al. (2001) showed that *P. chrysosporium* produce similar LiP activity despite the change in agitation speed and mode but the relative compositions of LiP isoforms differ significantly. Apart from agitation mode, Farrell et al. (1989) and Glumoff et al. (1990) also demonstrated nutritional condition cause the variation in LiP isoforms. The study reported six LiP isoforms in culture of *P. chrysosporium* BKM-1767 under nitrogen limited condition, however, only five LiP isoforms were found in carbon limited condition (Farrell et al., 1989, Glumoff et al., 1990). Gene encoding *lipA – lipJ* are the common LiP isoforms found in *Phanerochaete chrysosporium.* In the past decade, numerous new gene coding for LiP isoforms have expanded to other WRF such as *Ceriporiopsis subvermispora* (Fernandez-Fueyo et al., 2012), *Bjerkandera adusta*, *Ganoderma* sp., *Phlebia brevispora* (Ruiz-Duenas et al., 2013) and *Phlebiopsis gigantea* (Hori et al., 2014).

Apart from fungi, LiP enzyme has also been found in bacteria notably actinomycetes. The first bacteria LiP enzyme designated as ALiP-P3 was found in *Streptomyces viridosporus* strain T7A (Ramachandra et al., 1988). Since then, numerous actinomycetes such as *Streptomyces chromofuscus* A2, *Streptomyces albus* ATCC 3005, *Streptomyces* sp. and *Streptomce psammoticus* were also revealed with

production of extracellular LiP (Pasti et al., 1991, Antonopoulos et al., 2001, Tuncer et al., 2004, Niladevi and Prema, 2005). Le Roes-hill et al (2011) demonstrated that termite gut is a good source in isolating LiP producing bacteria, thirteen LiP producers, *Streptomyces* sp. strains were isolated from termite guts of *Amitermes hastatus* (Le Roes-Hill et al., 2011). Besides that, extracellular LiP activity was also observed in *Bacillus* sp., *Brevibacillus* sp. and *Ochrobactrum oryzae* isolated from another termite species (*Bulbitermes* sp. and *Cryptotermes brevis*) (Kamsani et al., 2016, Tsegaye et al., 2018). Apart from that, LiP producing ability were also reported in several bacteria strains from genera *Burkholderia, Rhizobium, Lelliottia, Enterobacter,* and *Raoultella* isolated from rotten wood in Qinling Mountain (Yang et al., 2017). In recent year, LiP also expressed in newly isolated bacteria such as *Bacillus amyloliquefaciens* and *Bacillus licheniformis* (Mei et al., 2020, Zhou et al., 2017).

Interestingly, bacterial LiP is also present intracellularly unlike most of the fungi that secret LiP extracellularly. Both *Pseudomonas desmolyticum* NCIM 2112 and *Bacillus* sp. VUS demonstrated production of LiP intracellularly and extracellularly (Kalme et al., 2007, Dawkar et al., 2008). Kalme et al. (2007) demonstrated similar LiP enzyme activity in both intracellular and extracellular crude enzyme extracted from culture of *Pseudomonas desmolyticum* NCIM 2112. The study also reported that intracellular LiP activity was associated with the addition of dye in the culture medium. The intracellular LiP activity was induced upon addition of dye Direct Blue-6 in the culture medium of *Pseudomonas desmolyticum* NCIM 2112. This phenomenon was in conjunction with other dye biodegradation study by using *Bacillus* sp. VUS and *Comamonas* sp. UVS (Dawkar et al., 2008, Jadhav et al., 2008). Dawkar et al. (2010) and Jadhav et al. (2008) proposed that the biodegradation of azo dyes were initiated with cleavage by LiP followed by the oxidation and reduction of metabolites by other oxido-reductase enzymes in the crude culture (Dawkar et al., 2010). This mechanism is also well supported by numerous dye degradation studies using purified LiP. Purified LiP from *Pseudomonas* sp. SUK1 and *Brevibacillus laterosporus* MTCC 2298 (Kalyani et al., 2011, Gomare et al., 2008) initiate symmetric cleavage on Methyl Orange whereas *Bacillus* sp. VUS showed asymmetrical cleavage on Reactive Orange 16 (Dawkar et al., 2009).

2.6.3.1 Properties of LiP

In general, LiP a glycoprotein that facilitates in hydrogen peroxide dependent oxidative lignin degradation process. Although there are extensive studies on exploration of LiP producing fungi and the focus had started shifting to bacteria in the past 2 decades, however, the study with purified LiP is still limited compared to other ligninolytic enzymes (Lac, MnP). According to the past literature (table 2.8), LiP is an acidic protein with pI around 3.1~ 5.0 and molecular weight of around 33-50 kDa for fungi. However, molecular weight of LiP from *Pleurotus ostreatus* and most of the bacteria were generally higher with the range from 55 – 86 kDa. Besides that, a 28kDa of LiP was also identified in the study on *Serratia liquefaciens*. As mentioned in the previous section, presence of more than one LiP isozyme in the culture medium is a common phenomenon for fungi but it is rare for bacteria. However, a thermophilic actinomycete, *Streptomyces thermovialaceus* revealed two monomeric acidic LiP denoted by P-3 and P-5 (Iqbal et al., 1994). Iqbal et al. (1994) demonstrated LiP P-3 (82 kDa) exhibit broader substrate range comparing to LiP P-5. Study on *P. chrysosporium*, *T. versicolor* also demonstrated that each purified LiP isoforms exhibit different physical and catalytic properties (isoelectric points, molecular weight and substrate specificity) (Johansson and Nyman, 1993). The difference in amino acid composition and N-terminal amino acid sequence proposed that each LiP isozymes were made up from different genes (Glumoff et al., 1990, Johansson et al., 1993).

Table 2.8 Previous studies on characterisation of LiP enzyme from fungi and bacteria

Microorganism	Enzymes	pH	Temperature (℃)	Substrate	Km (mM)	Vmax	Molecular weight (kDa)	pI	Reference
***Pleurotus pulmonarius* CPG6**	LiP	3.0	30	Veratryl alcohol	0.025	ND	40	ND	(Giap et al., 2022)
Phanerochaete chrysosporium	LiP	5.5	30	Veratryl alcohol	0.065	142.8 µmol	55	ND	(Vandana et al., 2019)
Phlebia radiata	LiP	ND	ND	ND	ND	ND	44	3.8-3.9	(Vares et al., 1995)
***Bjerkandera* sp. strain BOS55**	LiP	2.5-3.0	ND	Veratryl alcohol	0.059-0.089	13.9-15.9 U/mg	40-42	ND	(Ten Have et al., 1998)
Irpex lacteus	LiP	ND	ND	Veratryl alcohol	0.2338-0.3264	ND	41-44	4.6-5.0	(Rothschild et al., 2002)
***Ganoderma* sp.**	LiP	3.0	25	Veratryl alcohol	0.401	5.3 U/mL	ND	ND	(Adebayo-Tayo et al., 2016)
Loweporus lividus	LiP	2.6	24	Veratryl alcohol	0.0580	ND	40	ND	(Yadav et al., 2009)
Trametes versicolor	LiP	ND	ND	Veratryl alcohol	ND	ND	41-43	3.1-3.7	(Johansson and Nyman, 1993)
Penicillium decumbens P6	LiP	4.0	45	Veratryl alcohol	0.565	0.088 mmol/ mg min	46.3	ND	(Yang et al., 2005)

Phanerochaete chrysosporium	LiP	ND	ND	Veratryl alcohol	0.086 - 0.483	ND	38-43	3.3-4.7	(Farrell et al., 1989)
Phanerochaete chrysosporium	LiP	2.3-3.2	ND	Veratryl alcohol	0.083-0.200	ND	39.5-42.0	3.7-4.7	(Glumoff et al., 1990)
Phanerochaete chrysosporium	LiP	3.0	30	Veratryl alcohol	0.1000	15.2 U/mg	36	ND	(Zeng et al., 2013)
***Phanerochaete sordida* YK-624**	LiP	ND	ND	Veratryl alcohol	ND	ND	50	ND	(Sugiura et al., 2003)
Pleurotus ostreatus	LiP	4.0	40	Pyrogallol	0.013	0.42 µmol/s	72	ND	(Kang et al., 1993)
***Schyzophylum commune* IBL - 06**	LiP	5	35	Veratryl alcohol	0.460	388 mM/min	33 & 47	ND	(Asgher et al., 2012)
***Bjerkandera* sp.**	LiP-MnP Hybrid	3.0	ND	Veratryl alcohol	1.500	13 U/ mg	45	ND	(Moreira et al., 2006)

ND: Not determined

Most of the purified LiPs are active at acidic condition with optimal pH around 3. Purified LiP from *Acinetobacter* sp. SW 30 and *Acinetobacter calcoaceticus* showed extremely acidic pH condition with optimal pH 2 and 1 respectively. Moreover, LiP from *Acinetobacter calcoaceticus* and *Streptomyces virodosporus* demonstrated a narrow working pH whereby no appreciable LiP activity were shown at pH value above 2.0 and 5.0 respectively (Ghodake et al., 2009, Tien et al., 1986). The acidic pH optimum exhibited by LiP is related to its extraordinary ability in oxidising substrate of high redox potentials. At low pH, the redox potential in LiP-I which is known as compound I oxo-ferryl intermediate is sufficiently high to catalyse the oxidation of non-phenolic compounds since the redox potential increased linearly as pH decrease (Wong, 2009). On the contrary, Haq et al. (2016) and Iqbal et al. (1994) demonstrated optimal pH close to neutrality for LiPs from *Serratia liquefaciens* and *Streptomyces thermovialacues* when utilising veratryl alcohol and 2,4-DCP as assay substrate, respectively.

Thermostability is an important criterion in considering the potential of an enzyme in industrial application. However, fungal LiPs have lower thermotolerance relative to bacterial LiPs. *Penicillium decumbens* P6 and *Pleurotus ostreatus* attained optimum temperature at 45℃ and 40℃ respectively (Yang et al., 2005, Kang et al., 1993). Low thermotolerant properties of fungal LiP hinder the application of LiP in industrial lignin degradation process which often involves high temperature. Immobilization of enzyme is the recent biotechnology technique used to improve the stability of an enzyme (Asgher et al., 2014). Asgher et al (2007) proved that immobilization of *Phanerochaete chrysosporium* LiP in xerogels conferred considerable improvement on the thermal stability of LiP (Asgher et al., 2007). Similar case was also observed in *Coriolus versicolor* whereby the co-immobilised LiP-MnP by microspheres prolong the denaturation of LiP from 6 h to 24 h during incubation at 50 ℃. However, some thermophilic bacteria LiPs have greater thermostability than those immobilised fungal LiP. Both peroxidase from *Bacillus stearotgermophilus* and *Streptomyces thermoviolaceus* are highly stable whereby both LiP showed optimum temperature at around 70℃ (Iqbal et al., 1994, Loprasert et al., 1988). Moreover, 100% of activity was retained in *Streptomyces thermoviolaceus* LiP after incubating at 50℃ for 24 h.

LiP is able to catalyse oxidation process of phenolic through cleavage of the propyl side chain, demethylation, intermolecular addition or polymerisation. Guaiacol, ABTS, 2,6-DMP, vanillic acid, syringic acid and catechol are the most common phenolic substrate for LiP (Wong, 2009). *Irpex lacteus* LiP is capable to degrade guaiacol, 2,6-DMP and hydroquinone (Rothschild et al., 2002). Azo, heterocyclic, thiazine, polymeric dye was also found to be associated with LiPs from *Acinetobacter calcoaceticus*, *Kocuria rosea* and *Phanerochaete chrysosporium* (Ghodake et al., 2009, Parshetti et al., 2012, Ollikka et al., 1993). Azure B, a thiazine dye that is often used as an indicative substrate in screening LiP activity since it only can be degraded with high redox potential enzymes. In recent study, two fresh water bacteria, *Raoultella ornithinolytica* and *Ensifer adhaerens* were reported to have the ability in degrading Azure B dye, possibly due to the inclusion of dye in arene substituents by LiP (Falade et al., 2017).

Moreover, LiP is still able to catalyse oxidation of non-phenolic aromatic compounds with high redox potentials that are not readily oxidised by other ligninolytic enzymes through hydroxylation of benzylic methylene groups and oxidation of benzyl alcohols in the presence of H_2O_2 (Niladevi, 2009, Furukawa et al., 2014, Wong, 2009). Thus, this extraordinary ability plays an important role in lignin degradation since 90% of lignin is comprised of non-phenolic compound. This characteristic enables LiP to catalyse degradation of a wide range of non-phenolic compounds, such as veratryl alcohol, dimethylphenylenediamine and non-phenolic $\beta - O - 4$ linked arylglycerol β-aryl ethers (Wong, 2009, Valli et al., 1990) although LiPs were found to catalyse oxidation of phenolic compound more readily than nonphenolic one (Pollegioni et al., 2015, Chandra et al., 2017).

2.6.3.2 Catalytic Mechanism of LiP

Veratryl alcohol is an excellent substrate since it could act as the mediator to stimulate oxidation process for prevention of enzyme inactivation (Niladevi, 2009). Furthermore, veratryl alcohol could facilitate as a redox mediator for indirect oxidation of other substances as shown in Figure 2.5. The catalytic cycle of LiP is initiated through the reaction between H_2O_2 and native LiP enzyme to form compound I intermediate that exists as a ferry iron poryhyrin radical cation [Fe(IV)=$O^{\cdot+}$, LiP-I]. Subsequently, reducing substrate such as veratryl alcohol reduced with LiP-I to form LiP-II [Fe(IV)=O, LiP-II] and a VA cation radical ($VA^{\cdot+}$) via $1e^-$ reduction of LiP-I. A second $1e^-$ reduction transforms the LiP-II complex to the native enzymes. There is another alternative where LiP-II reacts with excess H_2O_2 in the presence of reducing substrate to form an inactive form LiP-III compound which exists as a ferric-superoxo complex [Fe(III)$O_2^{\cdot-}$]. Lastly, LiP-III is converted to the native LiP enzyme by oxidation with a VA radical.

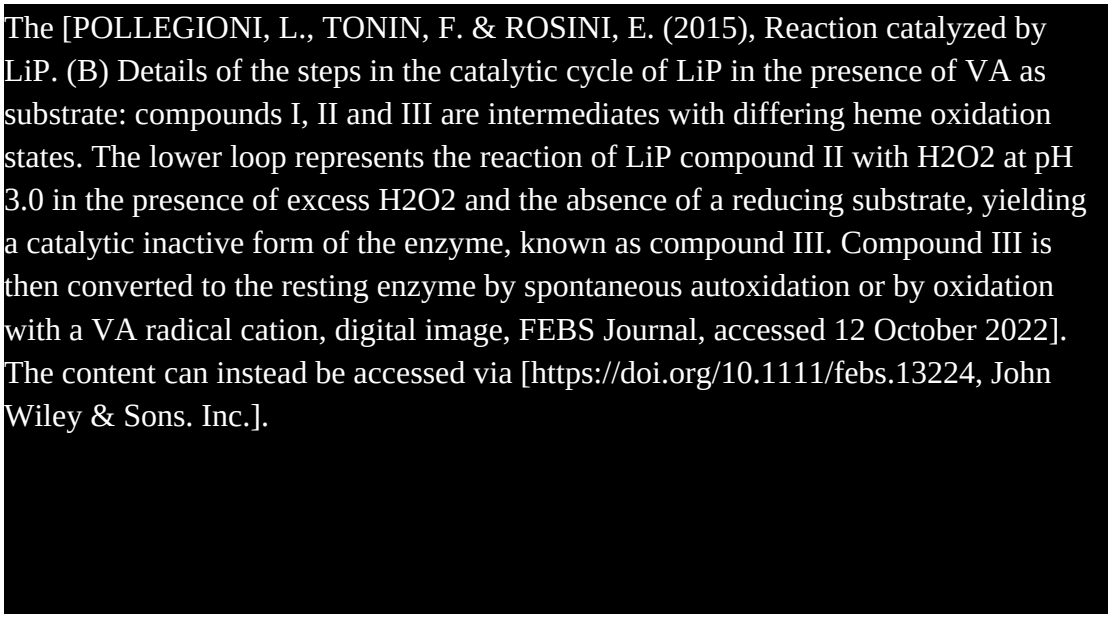


Figure 2.5 Catalytic pathway of LiP in the presence of VA as substrate (Pollegioni et al., 2015)

2.7 Enzyme production

Cost effectiveness is the main concern in industrialization of any process. Medium optimisation is a traditional method used to enhance the harvest of the enzyme production where the improvement of fermentation parameters can be achieved.

Extensive studies have been done on LiP production by lignin degrading fungi such as *Trametes versicolor*, *Schizophyllum commune Phanerochaete chrysosporium, Ganoderma lucidum* and *Endomelanconiopsis* sp. (Iqbal et al., 2011, Irshad and Asgher, 2011, Asgher et al., 2006, Batool et al., 2013, Nayana et al., 2020). These studies showed that fermentation conditions and nutritional parameters have significant effect on LiP enzyme activity. The fermentation conditions and nutritional parameters were varied quite considerably. The fermentation conditions are often referred to physical parameters of fermentation process such as fermentation configuration, pH, temperature, incubation period and inoculum size. As for nutritional medium, it involves the composition of nutrients such as carbon sources, nitrogen sources, trace metals, inducers, surfactants, and substrate concentration (Debnath and Saha, 2020). Optimisation of these abiotic conditions will improve the ligninolytic enzymes production and efficiency of enzymatic delignification process. The following factors affecting the ligninolytic enzymes production by fungi and bacteria have been studied in detail.

2.7.1 Fermentation technique

Fermentation is one of the primary techniques employed for production of enzyme. SSF and SmF are among the common fermentation techniques used in producing ligninolytic enzyme. SSF is a fermentation technique in which microorganism grow on solid substrate while involving nearly no moisture or low moisture condition (Pandey, 2003, Krishna, 2005). On the contrary, SmF utilises free flowing liquid medium as a substrate to support the growth of microorganism during the fermentation process (Subramaniyam and Vimala, 2012). Conventioanlly, SSF is preferred for fermentation involving fungi and microorganisms that require less moisture content, whereas SmF is best suited for fermentation process involving microorganisms that require high water activity such as bacteria (Manpreet et al., 2005, Subramaniyam and Vimala, 2012).

Studies by Niladevi and Prema (2005) and Niladevi, Sukumaran, and Prema (2007) demonstrated both SmF and SSF are also functional for bacterial enzyme production. 3.06 U/ml and 55.4 U/gds of laccase enzyme activity was observed in laccase production under SmF and SSF of rice straw using *Streptomyces* sp. respectively. In previous studies, most of the peroxidase from bacteria such as

Enterobacter hormaechei, *Bacillus licheniformis* and *Bacillus* sp. MABINYA-1 were produced through SmF technique (Zhou et al., 2017, Falade et al., 2020). In fact, SmF is the main method used in industry because it is easier to scale up for large production. Apart from that, it is easier in control of process parameters such as pH, temperature, substrate and production concentration and mixing speed. However, recent study showed that LiP enzyme from *Bulbitermes sp.* could be produced through SSF (Kamsani et al., 2016). Overall, SSF is a biotechnology process with low environmental impact, low cost, low energy requirements and less wastewater production which makes the downstream processes easier. However, scaling up of SSF remain challenges due to difficulties in control of process parameters which lead to large batch to batch variation. Indeed, previous comparative study on amylase, xylanase, cellulase and pectinase production confirmed that different fermentation technique has direct impact on enzyme productivity from *Bacillus* sp. (Subramaniyam and Vimala, 2012, Oumer and Abate, 2018). There is very limited investigation on comparative study of cultivation methods such as SSF and SmF on LiP production, thus, it is worth to determine the study the production of enzyme via SSF or SmF.

2.7.2 Physical parameters

Incubation period plays an important role in enzyme production through fermentation process. In general, the enzyme activity increased with increasing incubation period up to a certain extent. Longer incubation period than the optimal incubation period would lead to decrease in the enzyme production. This is probably due to nutrient limitations which might stress the microorganism physiology resulting in the inactivation of secretory machinery of enzymes (Falade et al., 2019b). Moreover, autolysis of the culture and denaturation of the enzyme could be the reason for the decreased enzyme activity (Wang et al., 1993, Tuohy and Coughlan, 1992). Additionally, some by-products produced along with the enzyme production process might cause inhibition on the growth of the microorganisms and enzyme production (Yee et al., 1996, Korus et al., 1991).

Inoculum size is a vital factor in enzyme production in both submerged fermentation and solid-state fermentation process since lower level of inoculum

density may not be sufficient to initiate the growth, thereby increasing the incubation period for the substrates. Besides, higher inoculum density may cause competitive inhibition in enzyme production due to increased spore concentration. Based on the studies reported, two forms of inoculum were used, namely. an agar plug and a liquid form. Patel, Gupte and Gupte (2009) maximised Laccase production by optimising the inoculum size in agar plug form. 5 agar plugs with 8 mm in diameter each was reported as the optimum inoculum size in producing Laccase enzyme by *Pleurotus Ostreatus* under SSF of wheat straw (Patel et al., 2009). Asgher, Sharif, and Bhatti (2010) have examined the effect of inoculum volume on ligninolytic enzymes production by G. lucidum-IBL-06 under SSF of rice straw. The examined range of inoculum volume is 3-7 ml with 1×10^7 conidia/ml. It was found out that 6 ml of inoculum is the optimum inoculum size for maximum LiP, MnP and Lac enzyme production (Asgher et al., 2010). A similar study was conducted by Hariharan and Nambisan (2013) using the same strain under SSF of pineapple leaf. A volume of 4 ml with 5×10^7 spores/ml was identified as the optimum inoculum size (Hariharan and Nambisan, 2013).

Incubation temperature of the medium is another major factor that influences the enzyme production. Incubation temperature will affect the survival rate of the microorganisms and production of active and stable enzymes. High temperature might cause denaturation of the microorganisms or distortion of the three-dimensional structure of the enzymes, in turn reduce the number of active enzymes. The optimum incubation temperature varies with the type of microorganisms. Most of the ligninolytic enzyme producing fungi are not thermophiles. The optimum temperature for ligninolytic enzyme production from fungi ranged from 25-35℃ (Asgher et al., 2011, Iqbal et al., 2011, Asgher et al., 2012). In recent years, *P. chrysosporium* IBL-03, *Schyzophyllum commune*, *Ganoderma lucidum* and *Trametes versicolor* were some common lignin degrading fungi that have been studied. The optimum incubation temperature for the strains above was at 35℃ except for *Trametes versicolor* at 30℃. Interestingly, Manimozhi and Kaviyarasan (2012) showed that the optimum incubation temperature for biomass production was different from optimum temperature of laccase activity under submerged fermentation from *Agaricus Heterocystis*. The optimum temperature for biomass production and laccase activity was found to be 30^{o}C and 25^{o}C respectively (Manimozhi and Kaviyarasan, 2012).

To date, there are very few studies on optimising bacterial lignin enzymes production. Most of the previous studies focused on α-amylase, cellulase and lipase enzyme production from bacteria. In general, the optimum temperature for facultative thermophilic bacteria range from 50-70 ℃ (Kikani et al., 2010). *Bacillus subtilis* and *Bacillus thermoleovorans* were employed for industrial α-amylase and lipase enzyme production with optimum incubation temperature reported at 70 ℃ and 60℃ $respectively$. Taylor et al. (2012) and Wang, Lin, et al. (2013) have isolated *Sphingobacterium* sp. from soil sample and activated sludge of pulp and paper mill respectively for lignin derivatives degrading purposes. These authors showed that both *Sphingobacterium* grew optimally at different temperature of 45 - 50℃ and 30℃ in lignin and lignosulphonate degradation process respectively.

Initial pH of the medium is a vital factor affecting enzymes production. The optimum pH for ligninolytic production is highly dependent on nutritional composition of the substrates and fermentation media. Bacteria growth is highly sensitive to pH since most of the bacteria only active in a specific pH condition. Any pH condition lower or higher than the optimum pH will yield low bacteria growth and enzyme production. In addition, low or high pH will cause distortion on the three-dimensional structure of enzymes which decrease the total number of enzymes' active sites. Furthermore, low number of active sites would cause low enzyme production. It was reported the optimum initial pH value ranges from 4 to 6 for most of the ligninolytic enzyme production from fungal (Asgher et al., 2012). LiP, MnP and Lac productions were maximised at pH 4.5 from both *P. chrysosporium* IBL-03 and *Schizophyllum commune* IBL-06 in SSF from banana stalks (Asgher et al., 2011, Irshad and Asgher, 2011). However, maximum peroxidase production was observed at pH 8 for actinomycetes *Streptomyces* sp. strain (Musengi et al., 2014).

2.7.3 Nutritional parameters

To date, there are extensive studies on using various carbon sources in enhancing ligninolytic enzymes (Kanwal and Reddy, 2011, Hariharan and Nambisan, 2013, Dhakar and Pandey, 2013). Glucose, sucrose, starch, maltose and lactose are the common carbon sources used in enzyme production. Hariharan and Nambisan (2013)

have performed a single factor optimization in examine the effect of various carbon sources on ligninolytic enzymes production from *Ganoderma lucidum*. In this study, Lignin Peroxidase, Manganese Peroxidase and Laccase were produced by *Ganoderma lucidum* under SSF from pineapple leaf. According to the result, only glucose and sucrose showed increased ligninolytic enzymes production. The result showed that 1% (w/w) of sucrose increased enzyme activity from 1350 IU/mL LiP, 680 IU/mL MnP and 300 IU/mL Lac to 2098.3 IU/mL LiP, 810.9 IU/mL MnP, and 425.8 IU/mL Lac respectively. In comparison with control, at least 50% of decrement in enzyme yield was observed in the media with addition of starch, maltose and lactose as carbon source. However, no explanation was given by Hariharan and Nambisan (2013) on this phenomenon.

In 2011, Kanwal and Reddy have examined 12 different types of carbon sources in producing Laccase enzymes by *Morchella crassipes*. The carbon sources tested including Glucose, Fructose, Sucrose, Mannose, Cellobiose, Ribose, Rhamnose, Galactose, Arabinose, Xylose, Sorbose and Mannitol. Based on the growth of dry biomass, maximum growth was observed in the media containing glucose, fructose and mannitol. However, a different trend was observed in the laccase enzyme production. Media with addition of mannose, rhamnose and galactose showed higher laccase activities as compared to other carbon sources. The highest activity was with mannose at 3.48 U/mL of laccase activity.

Another similar study on Laccase production was reported by Dhakar and Pandey in 2013. This study aimed to investigate the effect of nutritional parameters with different carbon sources on Laccase production by *Trametes hirsute* (MTCC 11397). There was total six different carbon sources (glucose, fructose, maltose, sucrose, starch and cellulose) being examined, and glucose was set as the control experiment. Among all carbon sources, fructose was found to increase the production of laccase along with higher protein content and reduced fungal biomass, in comparison to Glucose. Based on the single factor optimisation result, the fungal biomass and protein content increased as the concentration of fructose increase from 0.0% to 1.0%. Nevertheless, decreased laccase activity was observed as the fructose concentration exceeded 0.4%. The maximum laccase activity was reported as 435.0 U/L at 0.4% of fructose. The protein content, biomass growth and laccase activity were inhibited by other carbon sources (maltose, sucrose, starch and cellulose), especially

cellulose. Dhakar and Pandey (2013) suggested this may be attributed to the specific preference for the carbon source by the *Tramates hirsuta* (MTCC 11397).

2.7.4 Nitrogen source

In the past studies, various types of inorganic and organic nitrogen sources have been examined extensively. These nitrogen sources included beef extract, peptone, yeast extract, ammonium sulphate, ammonium nitrate, urea and potassium nitrate (Kachlishvili et al., 2006, Iqbal et al., 2011, Kanwal and Reddy, 2011, Dhakar and Pandey, 2013, Hariharan and Nambisan, 2013). Kachlishvili et al. (2006) have carried out some parametric study on using Potassium nitrate, Ammonium sulphate, Ammonium nitrate and peptone as nitrogen sources in the Solid State Fermentation of beech tree leaves and wheat straw respectively. In this study, effects of these various nitrogen sources on fungal lignocellulolytic enzyme activities were investigated. *P. dryinus* IBB 903, *P. tuberregium* IBB 624, *L. edodes* IBB 363 and *F. trogii* IBB 146 were the fungi involved in the ligninolytic enzyme production. Based on the result, effect of nitrogen sources on the ligninolytic enzymes production is strain dependent. Ammonium nitrate was reported as the suitable nitrogen source in enhancing Laccase production by *L. edodes* IBB 363 and *F. trogii* IBB 146 under both beech tree leaves and wheat straw in SSF. The laccase production was increased by two-fold with the addition of Ammonium nitrate as compared to control experiment. Besides this, Kachlishvili et al. (2006) reported that Potassium nitrate, Ammonium sulphate, Ammonium nitrate and peptone did not significantly affect and enhanced the Manganese Peroxidase by the fungi strains above. This was probably due to low Manganese Peroxidase activities in the fungi strains. However, Kanwal and Reddy (2011) reported in their study with *Morchella crassipes* showed that only Laccase activity was observed in the media tested with different nitrogen sources. They examined seven types of nitrogen sources which included Ammonium nitrate, Sodium nitrite, Sodium nitrate, peptone, yeast extract, casein and Tryptone in this study. Among the nitrogen sources tested, casein and peptone were identified as the best nitrogen sources for the laccase production by *M. crassipes*. Furthermore, laccase production in casein and peptone was almost two fold of that produced in inorganic nitrogen sources (Kanwal and Reddy, 2011).

Similar study was conducted by Iqbal, Asgher and Bhatti (2011) in examining the stimulatory/ inhibitory effects of carbon and nitrogen source combination on ligninolytic enzymes production by *Trametes versicolor* under pre-optimized conditions. However, different nitrogen sources were considered in this study including urea, yeast extract, beef extract, peptone and ammonium sulphate. The result showed that combination of glucose and peptone, maltose and urea were the best for both Lignin Peroxidase (1576 U/mL) and Manganese Peroxidase (1542 U/mL), while glucose and yeast extract was the best for Laccase (98.05 U/mL) production. These observations suggest that nitrogen source is a vital aspect in regulating the ligninolytic enzymes production.

Besides this, similar study was reported by Hariharan and Nambisan (2013) on effect of nitrogen sources on ligninolytic enzymes production by *Ganoderma lucidum*. In this study, urea, yeast extract, beef extract, peptone, ammonium nitrate and ammonium sulphate were the nitrogen sources. The result showed that all nitrogen sources were able to enhance the ligninolytic enzymes whereby the productions were comparable or higher than the control experiment except for Ammonium Sulphate. Supplying Ammonium sulphate showed inhibition on Lignin Peroxidase, Manganese Peroxidase and Laccase production. Among these nitrogen sources tested, addition of beef extract have shown significant enhancement on the ligninolytic enzymes, particularly on Lignin Peroxidase production (Hariharan and Nambisan, 2013).

2.7.5 Substrate concentration

Currently, lignocellulosic materials are the common substrates used for bioethanol production. Most of the studies were focused on utilising natural, agricultural (wheat straw, rice straw, sugarcane bagasse) and agro-industrial wastes (corn cob, pineapple leaf, orange waste) (Champagne, 2008, Braide et al., 2016, Chintagunta et al., 2017, Orozco et al., 2014). However, kraft lignin is the common substrate used to explore the potential of lignin degradability and ligninolytic enzyme production in a newly isolated strain. Nandal, Ravella, and Kuhad (2013) demonstrated that substrate concentration has great influence on laccase enzyme production. Moreover, it has been reported that LiP production in *Trametes trogii*, *Nonomuraea gerenzanensis* and *Pseudomonas* sp. strain Y6 was stimulated in the presence of lignin (Levin and Forchiassin, 2001, Casciello et al., 2017, Yang et al., 2017). Moreover,

Streptomyces sp. S6 was able to utilise kraft lignin as sole carbon source in LiP production (Riyadi et al., 2020). However, *Phanerochaete chrysosporium* and *Phlebia radiata* was also able to induce LiP enzyme in the absence of kraft lignin or any wood containing carbon source (Wang et al., 2008, Rogalski et al., 1991). Similarly, addition of lignin also showed negative effect on peroxidase production by *Streptomyces chromofuscus* A2 and *S. viridosporus* T7A in glucose containing F medium (Pasti et al., 1991). Thus, it is noted that the lignin concentration is certainly affecting the LiP production. However, the investigation on optimal lignin concentration on LiP production is relatively scarce particularly on thermophilic bacteria. Hence, further study is required to establish the relationship between substrate concentration with LiP enzyme production by bacteria.

2.8 Lignin degradation application

Lignin degradation is an important step in second-generation (2G) biofuels, pulp and paper making industry and composting process (Biko et al., 2020). Ligninolytic enzyme also serve as bioremediation agent in paper mill effluent and textile wastewater. Many of these industries use thermochemical and chemical method for depolymerisation of lignin or derived lignin compound which involves harsh conditions and produce toxic by-products. These methods are less cost effective since extra step is needed to remove toxic by-products. Apart from that, many of these industries work at thermophilic or alkali conditions which may cause denaturation of enzyme sourced from fungi. Thus, the use of thermophilic LiP from bacteria may help to overcome these challenges due to its greater thermal stability and broad working pH even at alkali condition.

2.8.1 Biofuel production

Microbial biodegradation of lignin has become an important pretreatment in biofuel production from lignocellulosic material such as bioethanol and biodiesel. In bioethanol and biodiesel process, cellulose and lignin need to be segregated to enrich fermentable carbohydrate for following ethanol and diesel production. This step is essentially important because amount of cellulose that is available for biofuel

production is dependent on the effectiveness in lignin removal. The bacterial ligninolytic enzymes produced by K. pneumonia has significantly enhanced 39.3% of bioethanol yield from wheat and rice bran compared to non-enzyme treated wheat and rice bran(Gaur et al., 2018).

2.8.2 Paper and pulp industry

In the paper and pulp industry, cellulose and lignin need to be separated to produce high quality paper. Conventionally, chemical is used to pretreat the paper making feedstock before it is pulped for mechanically or physically for paper production. A recent approach involves pretreat the wood used to produce paper with microbes or their ligninolytic enzymes consortium in a process called biopulping (Atiwesh et al., 2022). Wang et al. (2013) reported bacteria consortium named LDC that made up from *Paenibacillus* sp., *Microbacterium* sp., *Pseudomonas* sp. and *Acinetobacter* sp. can degrade 60.9% lignin in reed straws that used as feedstock for paper making. The paper sheets produced from LDC pretreated reed straws displayed higher breaking length and tensile index compared to chemical pretreated LDC. The improvement in the physical properties of paper sheets inferred biopulping is effective in pretreating feedstock for paper and pulp process (Wang et al., 2013b).

2.8.3 Bioremediation and detoxification

During the production of pulp and paper from plant material, large amount of effluent which contain high amount of lignin, collectively named as black liquor is being generated and discharged into the environment. The discharged of untreated black liquor caused severe aquatic and soil pollution. The toxic compound, kraft lignin endanger the survival of aquatic flora and fauna by limiting the transmission of sunlight and oxygen availability in water (Chauhan, 2020). Numerous bacteria such as *Azobacter*, *Serratia*, *Bacillus* and *Pandoraea* sp. have been reported which have decolorisation ability and decrease the toxicity in black liquor. The bioremediation or treatment of black liquor often involve alkali and high temperature conditions. Thus, the use of thermophilic and alkaliphilic bacteria for bioremediation of black liquor may be potentially useful and effective in treating lignin and toxic compound. An et al. (2021) reported a thermophilic and alkali strain *Serratia* sp.AXJ-M achieved a 70% of

lignin removal rate after 7 days of incubation under optimal conditions (An et al., 2021).

Apart from pulp and paper mill effluent, dye containing wastewater from textile industry also contributed to environment pollution. Azo dyes which represent the major group of dye used in the textile industry. Azo dyes has similar lignin structure that often resist degradation process. Lignin peroxidase is known to play an important role in biodegradation of azo and other textile dye. The working pH of fungi peroxidase is acidic, whereas bacteria peroxidase has broader working pH from acidic to alkali. Thus, application of bacteria ligninolytic enzyme can be further extended to dye decolourisation industry that work at neutral pH or slightly alkali condition (Kishor et al., 2021, Guo et al., 2020). Several bacteria from genus Bacillus have been used to decolorise synthetic dye. A Bacillus sp. React3 LiP decolorised 99% of MB at pH 7 within 48 h (Kim et al., 2022). Guo et al (2021) reported a decolorising efficiency of 92.6% with LiP producing bacteria consortium that made up from *Bacillus* and *Piscibacillus* after 48 h treatment of metanil yellow G (MYG) at 50℃ (Guo et al., 2021).

2.8.4 Composting

Lastly, the lignin pretreatment process can be applied in composting process to shorten the composting period and improve the quality of the final compost. Composting is the natural biological degradation recycling of organic matter by a group of microorganisms such as fungi, bacteria and actinomycetes into fertiliser that can enrich soil. It is commonly prepared by decomposing lignocellulosic plant waste, food waste or manure under aerobic conditions, controlled moist and oxygen level. This composting process take places in four phases according to temperature pattern: initial activation phase, mesophilic phase, thermophilic phase, and maturation phase. Intense decomposition of organic matter is mainly occurring at thermophilic temperature through oxidation by various of ligninolytic and hemicellulolytic enzymes (Hemati et al., 2021). A thermotolerant alkali lignin degrading strain *Aneurinibacillus* sp. LD3 promoted lignin degradation rate at 61.28% in food compost and increase the formation of aromatic substance and humic-like substance (Wu et al., 2022). Similarly, Hemati et al (2022) also demonstrated the use of thermophilic isolate was able to accelerate the composting process and increase the total acidity in humic acid extracts

(phenolics, quinones, ketones, and carboxylic groups) which contain nutrients that is essentially useful to plant roots and improving soil fertility (Hemati et al., 2022). This showed that addition of ligninolytic microbes not only improve the qualitative properties of compost, it also shorten the composting period by increasing the degradation rate at thermophilic phase.

Chapter 3 Methodology

3.1 Methodology Overview

In this research study, the methodology is divided into 4 sections which include screening and isolation of thermophilic ligninolytic bacteria, cultivation of selected strains using SmF and SSF, optimal growth condition for LiP enzyme production and characterisation of LiP. A simple flowchart on methodology is illustrated in Figure 3.1.

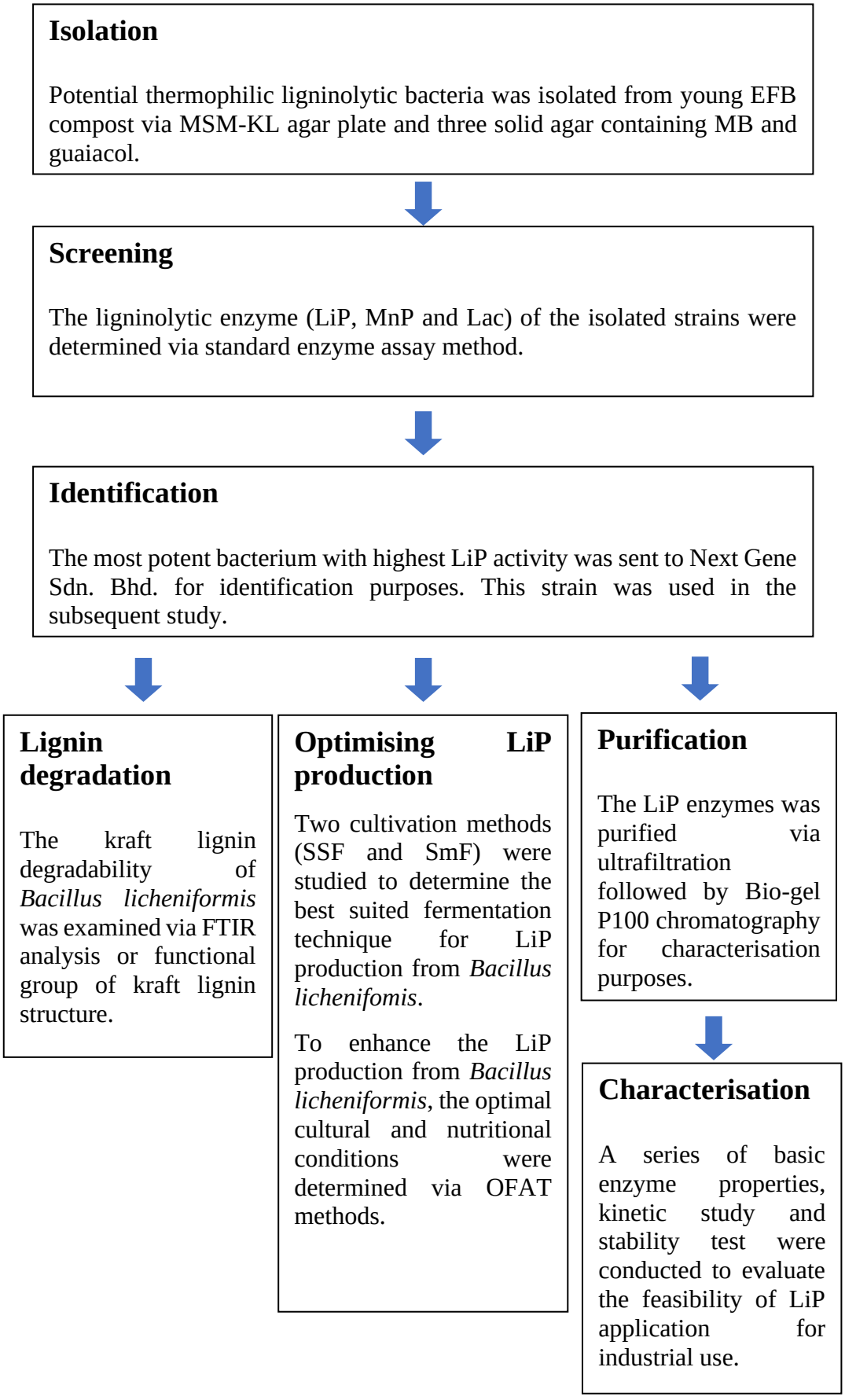


Figure 3.1 Overview on methodology in this study

3.2 Materials

All chemicals used in this study were of analytical grade. Kraft lignin, K_2HPO_4, KH_2PO_4, $CaCl_2$, $MgSO_4$, NH_4NO_3, $CuSO_4$, $FeSO_4$, ZnSO4, Yeast extract powder, glucose, tryptic soy agar, sucrose, tryptic soy broth No. 2, veratryl alcohol, citric acid, Na_2HPO_4, sodium lactate, lactic acid, ABTS, sodium acetate, acetic acid, bovine serum albumin (BSA), 2,6-dimethoxyphenol, Azure B, HCl, SDS, EDTA, Tween-80, NaN_3 were purchased from Sigma-Aldrich (USA). $MnSO_4$, 30% H_2O_2, Gram-colour stain set, immersion oil, NaOH, NaH_2PO_4, $FeCl_2$, $FeCl_3$, $MgCl_2$, K Cl, NaCl, $MnCl_2$, $NiCl_2$ were purchased from Merck (Germany). Agar powder, Malachite Green, Phenol Red were purchased from Bendosen. Guaiacol and Tris(hydroxymethyl) aminomethane hydrochloride were purchased from Acros Organics (Belgium). Chromatein prestained Protein ladder (PR0602) was purchased from Vivantis Technologies Sdn. Bhd. (Malaysia). Methylene blue was purchased from Fisher Scientific (UK). Ethanol absolute (99%) was purchased from HmbG (Germany).

The following instruments were used in the study: incubator with natural convection BD240L (Binder); digital shaking incubator 311DS (Labnet), Ulpasafe biosafety cabinet 4IIA (SASTEC), Autoclave HVE-50 (Hirayama), Eclipse E200 LED microscope (Nikon) with Toupcom 5MP UCMOS camera, FreeZone 2.5 Litre Benchtop Freeze Dryers (Labconco), Sension +pH 1 portable pH meter (HACH), Universal 320R centrifuge (Hettich), UV-vis spectrophotometer (Perkin Elmer), Cary 630 Fourier Transform Infrared Spectroscopy (Agilent), Amicon 8050 dead end stirred cell (50 mL) (Millipore), Zetasizer Nano-ZSP with autotitrator MPT-2 (Malvern Panalytical) and Waterbath WNB14L (Memmert).

3.3 Sample collection and isolation of thermophilic bacteria

Young EFB compost from day 10, day 12 and day 14 were obtained from a local composting site in Miri, Malaysia. These young EFB compost were used as the source for isolation of thermophilic ligninolytic bacteria. Five gram of EFB compost were re-enriched with 95 mL Minimal Salt Medium containing 0.5 g/L of kraft lignin (MSM-KL) in 250 ml Erlenmeyer flasks under SmF. The Minimal Salt Medium contained (g/L): KL (0.5); K_2HPO_4 (4.55); KH_2PO_4 (0.53); $CaCl_2$ (0.5); $MgSO_4$ (0.5);

NH_4NO_3 (5) with trace elements $CuSO_4$ (0.002), $FeSO_4$ (0.01); $MnSO_4$ (0.001) and $ZnSO_4$ (0.001). As suggested by Zainuddin et al. (2013), the MSM-KL containing young EFB compost was incubated at thermophilic temperature 50℃ in incubator with natural convection (Brand: Binder, Model: BD240L) for 7 days at stationary condition for enrichment purpose (Zainudin et al., 2013). After 7 days of incubation, the incubated medium was agitated at 120 rpm in digital shaking incubator (Brand: Labnet-USA; Model: I5311-DS) for 30 min. The culture obtained was serially diluted with sterile distilled water until dilution of 10^{-6}. Hundred microliters of each diluted solution was plated on the MSM-KL agar plate (3.0 % w/v agar powder) using spread plate method (Abdul Rahman et al., 2013).

3.4 Screening of thermophilic bacteria with ligninolytic activity

The streak plate method was employed in isolating bacteria with fresh MSM-KL agar plates. Isolates that grew on the above agar plates were selected and re-streaked onto fresh agar plates and incubated at 50℃ for at least three days. Re-streaking process was repeated until pure colonies were obtained. The following three media were used to screen the ligninolytic ability of the pure colonies. (1) MSM-KL agar plate assays with 25 mg/L of Methylene Blue (MB) dye, 2.5 g/L Yeast extract, 2 g/L glucose; (2) Tryptic Soy Agar (TSA) with 25 mg/L of MB, (3) 1 mM Guaiacol, a lignin mimicking compounds was used as a carbon source in MSM-KL agar plate assays (Niladevi and Prema, 2005). For plate assays, agar plates were incubated at 50℃ for seven days. Growth and decolourization of the agar was monitored daily. Pure colonies were subjected to Gram staining and observed under a microscope. Selected isolates were maintained on TSA plate at 4℃ and freeze-dried according to the following steps. Ten gram of sucrose was dissolved in 100 mL of deionized water and sterilised by filtration through 0.22µm filters. The selected isolates were harvested directly from agar plates after 3 days and mixed with 10% (w/v) of sterile sucrose solution. Bacteria suspension in the protectant medium were stored into three 2 mL vial. The vials were placed in freeze dried sample flask and frozen at -80°C for overnight. The pre-frozen flask was freeze dried using FreeZone Benchtop Freeze Dryers (Labconco; Model: 2.5 Liter -50℃) under Auto Mode (-50℃, vacuum level <0.133 mBar) for overnight. The freeze-dried isolate samples were stored in Ultra Low Freezer (Brand: CLIMASLAB; Model: CVF 220/86) at -80℃.

3.5 Enzyme profile of the selected isolates

Ten isolates which decolourise or showed good growth were selected and grown aerobically in 80 mL MSM-KL medium with 10 g/L glucose, 1.0 g/L yeast extract and addition of 4% (v/v) inoculum in 250 mL Erlenmeyer flasks under submerge fermentation at 50℃ for 120 h (Buraimoh et al., 2015). Slight modification were made in inoculum size to boost the LiP production. Submerged fermentation was selected since this is commonly used in ligninoltic enzyme production by bacteria such as *Bacillus* sp. and *Streptomyces* (Abd-Elsalam and El-Hanafy, 2009, Abdul Rahman et al., 2013, Buraimoh et al., 2015). Inoculum was prepared by inoculating one loopful of each selected isolate into 20 ml of Tryptic Soy Broth (TSB) and incubated at 50℃ for 16 h and dilute the inoculum concentration to final OD 600 of 1.0 for subsequent growth study. An un-inoculated MSM-KL medium was served as control and incubated under the same condition. 5 mL of culture samples were removed at 24 h intervals and tested for its crude enzyme activity including LiP, MnP and Lac.

3.5.1 Enzyme assay

Each culture samples were centrifuged at 4,000 rpm for 20 min at 4℃. The supernatant obtained after centrifugation (Brand: Hettich Universal 320R) was used for the enzyme assays.

3.5.1.1 LiP assay

The lignin peroxidase (LiP) activity was measured via the oxidation of veratryl alcohol to veratryaldehyde at 310nm. About 1.04 ml of enzymatic assay consisted of 0.4 mL of citrate-phosphate buffer (100 mM, pH 2.7), 0.1 mL of veratryl alcohol (20 mM), and 0.5 mL of fluid sample. Forty microliters of H_2O_2 (20 mM), which were freshly prepared daily, was added to initiate the reaction (Tien and Kirk, 1988). The conversion to veratraldehyde was monitored in 1 mL quartz cuvette (1 cm light path) at 310 nm wavelength using UV-vis spectrophotometer. One unit of enzyme activity corresponds to the oxidation of 1 micromole veratraldehyde converted from veratryl alcohol per minute under the assay conditions with molar extinction coefficient of $\epsilon 310 = 9300\ M^{-1}cm^{-1}$. The enzyme activity was expressed in U/L. The enzyme activity was calculated using equation 1.

$$Enzyme\ activity\ \left(\frac{U}{L}\right) = \frac{\Delta A/min}{\varepsilon \times b} \times \frac{V_t}{V_s} \times 10^6$$ *Equation 1*

$\Delta A/min$= average absorbance change per minute
V_t= total assay volume in ml
V_s= volume of sample in ml
ε= molar absorptivity of product
b= light path
10^6= factor to convert mole to micromole

3.5.1.2 MnP assay

Manganese peroxidase (MnP) activity was measured via the oxidation of guaiacol to a coloured product using UV-Vis spectrophotometer at 465nm. The enzymatic assay of 1.0 mL consisted of 0.4 mL of sodium lactate buffer (100mM, pH 4.5), 0.1 mL of guaiacol (final concentration: 1 mM), 0.1 mL of $MnSO_4$ (final concentration: 1 mM), and 0.4 mL of supernatant of the sample (Li et al., 2009). 30μL of H_2O_2 (final concentration: 0.1 mM) which was freshly prepared daily were added to initiate the oxidation of guaiacol. The formation of the coloured product was measured in the 1 mL quartz cuvette at 465 nm wavelength. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 micromole of coloured product per minute at 30℃ under the assay conditions with a molar extinction coefficient of $\epsilon 465 = 12100\ M^{-1}cm^{-1}$. The enzyme activity was expressed in U/L.

3.5.1.3 Lac assay

Laccase activity was assayed via oxidation of 2,2'-azinobis-(3-ethylbenzethiazoline-6-sulphonate) (ABTS) using method developed by Wolfenden and Wilson (1982). This enzymatic mixture made up of 0.15 mL of 0.03% ABTS, 0.5 mL of 0.1 M sodium acetate buffer at pH 5.0 and 0.35 mL of supernatant of the sample (Wolfenden and Willson, 1982). The formation of oxidized ABTS was measured in 1 mL quartz cuvette (1 cm light path) at 30℃ and 420 nm wavelength using UV-Vis Spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 micromole of coloured product per minute at 30℃ under the assay conditions with a molar extinction coefficient of $\epsilon 420 = 36\ 000\ M^{-1}cm^{-1}$. The enzyme activity was expressed in U/L.

3.5.2 Morphology of the isolates

Pure colonies were subjected to Gram staining and the morphology and size of the isolates were observed under microscope. Gram-colour stain set (Merck Millipore, Cat. No. 1.11885.0001) was used and the bacteria smear samples was stained according to standard protocol provided by the manufacturer. A drop of sterile water was placed on top of a glass slide. Bacteria colonies were transferred from the agar plate using the inoculation loop and a thin layer was smeared on top of the glass slide in circular motion starting from centre towards the outer area. The bacteria smears were heat-fixed with a Bunsen burner after they were fully air-dried in the Biosafety cabinet. The bacteria smears were then dropped and covered completely with Reagent 1: crystal violet solution for approximately 1 min and subsequently rinsed briefly with Reagent 2: Lugol's solution to remove excess crystal violet solution. Sufficient amount of Lugol's solution was used to cover the smear for about 1 minute and washed with distilled water for about 5 seconds. Tilt the glass slide in 45 upright positions with the smear facing up; dripped the Reagent 3 or 4: decolorising solution and carefully swirl the slices until no further cloud of dye was formed. Distilled water was used to rinse off the excessive amount of decolourising solution for about 5 seconds and smear was then covered with Reagent 5: Safranine solution for 45 seconds. Lastly, distilled water was used to wash off the Safranine solution and it was left to air-dry under room temperature condition.

The stained bacteria smears were viewed under laboratory microscope (Nikon) E200 LED MV series under 1000 x magnification and their images were snapped using the attached Toupcam 5MP UCMOS camera.

3.6 Bacteria identification

3.6.1 DNA extraction

The genomic DNA from the selected bacteria was extracted using Zymo Quick-DNA^{TM} Fungal/ Bacteria kit (Zymo Research) according to standard protocol

provided by manufacturer. BashingBead™ beating method and Zymo-Spin™ technology were employed to lyse the bacteria cell without addition of denaturants or proteinase, isolate and purify DNA that was readily used as PCR template.

3.6.2 Polymerase Chain Reaction (PCR) and 16S rRNA

The isolated DNA was subjected to polymerase chain reaction (PCR) amplification with the aid of universal primer: forward primer 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer 1492R (5′ CGGTTACCTTGTTACGACTT 3′). The cycler condition used for amplification of 16S rRNA gene consist of, 3 min of denaturation at 94℃; 30 cycles of 30 s at 94℃, 30 s at 50℃, 2 min at 72℃; followed by final extension of 5 min at 72℃. The PCR product was analysed on 1.0 % (w/v) agarose gel and sequencing was done by Next Gene Scientific Sdn. Bhd., Puchong, Selangor, Malaysia. The sequence was obtained using Sanger sequencing technology with the aid of four sequencing primers (27F, 518F, 800R, 1492R). The sequences were assembled by assembly program BioEdit 7.2.0 software.

3.6.3 Phylogenetic tree

Thereafter, nucleotide sequence of 16S rRNA gene was analysed using BLAST (Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). Multiple nucleotide sequence alignment was performed and aligned by CLUSTALW using Molecular Evolutionary Genetics Analysis software (MEGA) version 6.0. Phylogenetic tree was constructed by using neighbor-joining method with a bootstrap value of 1000 replicates to show the evolutionary relationships among the closely related bacteria available at the NCBI database.

3.7 Cultivation of selected strains using SmF and SSF

3.7.1 Inoculation preparation

Inoculum was prepared by obtaining one loopful of each isolate and inoculated into 20 mL of Tryptic Soy Broth (TSB) medium at an initial concentration of 10^7 cells/ mL. Samples were incubated at 50℃ with shaking at 120 rpm for 10 h to obtain a final OD_{600} of 1.0 as described by Abdul Rahman et al. (2013). After 10 h of incubation, the inoculum of *B. licheniformis* was situated at late growth phase and started entering the stationary growth phase. The growth profile of *B. licheniformis* in TSB medium was attached in Appendix B (Fig. B1).

3.7.2 MSM-KL media preparation

Medium used for both SSF and SmF was prepared as below. The MSM-KL media contained (g/L): KL (0.5); K_2HPO_4 (4.55); KH_2PO_4 (0.53); $CaCl_2$ (0.5); $MgSO_4$ (0.5); NH_4NO_3 (5) with trace elements $CuSO_4$ (0.002), $FeSO_4$ (0.01); $MnSO_4$ (0.001) and $ZnSO_4$ (0.001). The media was adjusted to pH 7.0 with 1M NaOH.

3.7.3 LiP production via solid state fermentation

For SSF, 8.45 mL MSM-KL media was added into 250 mL Erlenmeyer flask containing 2g of untreated EFB. The flask was added with 1 mL of 10 h inoculum (final OD_{600} of 1.0) (80% wet basis). The sample was incubated for 96 h in convection oven at 50℃, pH 7 under static and aerobic condition (Hamisan et al., 2009). Four flasks were prepared and each flask was withdrawn every 24 h to determine its LiP activity according to standard assay method mentioned in section 3.3.1.1. Hundred millilitre of sterile pure water was added into the flask for enzyme extraction. Five millilitre of culture sample was withdrawn and centrifuged at the speed of 4000 rpm using centrifugation machine (Brand: Hettich Universal 320R). The supernatant was then used in the following enzyme assay.

3.7.4 Submerged fermentation cultivation

For SmF, 2g of untreated EFB is mixed with 100 mL MSM-KL media with inoculum size of 4% (v/v). This fermentation was carried out in 250 mL Erlenmeyer flask for 96 h at 50℃ and pH 7 (Hamisan et al., 2009). Five millilitre of culture samples were collected at every 24 h interval. The collected culture was centrifuged at the speed of 4000 rpm before used for enzyme assay. The cultivation method which produced greater amount of crude ligninolytic enzymes activity was selected as the fermentation process in enzyme production. Standard enzyme assay was used to measure LiP enzyme activities (Li et al., 2009, Tien and Kirk, 1988, Wolfenden and Willson, 1982).

3.8 Optimal crude LiP production

In this parameter study, one factor at a time (OFAT) optimisation method was selected to study effect of various factors that is affecting the crude LiP enzyme production during fermentation process. The purpose of selecting OFAT because of its suitability to determine the efficient range of each parameters in producing LiP production for newly isolated bacteria strain. The studied factors including incubation time, temperature, inoculum, pH, glucose concentration, yeast extract concentration, lignin concentration and $CaCl_2$ concentration. OFAT was used to determine the parameters that affect the enzyme production and their efficient ranges of each parameter. All experiments were performed in triplicate to ensure reproducibility of results.

3.8.1 Effect of incubation time on crude LiP production

Bacillus licheniformis CLMTCHB29 was cultured in 50 ml of MSM-KL medium supplemented with 1.0 % (w/v) of glucose and 0.25 % (w/v) of yeast extract with 4% (v/v) inoculum. The medium was grown under 50℃ in static condition for 25 hours with initial pH 7. The culture sample was withdrawn at every 5 hours interval. The studied incubation time was adjusted accordingly to the result obtained from the study investigated in section 3.5.3. The culture sample was centrifuged (4000 rpm for 10 min, 4℃) and the LiP enzyme activity in crude supernatant was assayed using

standard LiP enzymes assay and the growth of biomass was measured at OD_{600}. All experiments were done in triplicate.

3.8.2 Effect of temperature on crude LiP production

Temperature is one of the critical factors in any fermentation process. *Bacillus licheniformis* is a thermophile and thus the optimum growth temperature was examined at thermophilic range as suggested by Nour El-Dein (2014) with slight modification to fit into efficient temperature range for *Bacillus licheniformis* CLMTCHB29. *Bacillus licheniformis* CLMTCHB29 was grown in 50 ml MSM-KL and medium supplemented with 1.0 % (w/v) of glucose and 0.25 % (w/v) of yeast extract with 4% (v/v) inoculum. The MSM-KL medium was initially adjusted to pH 7. The flask was incubated in temperature ranged from 45℃ to 55℃ and incubated for 15 h (preoptimized incubation time) under static condition. The culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity was determined using standard LiP enzyme assay stated in section 2.3.1.1. All experiments were done in triplicate.

3.8.3 Effect of inoculum size on crude LiP production

To examine the effect of inoculum size on the crude LiP production in MSM-KL culture medium (pH 7), the range of inoculum size was set as suggested by Jiang et al. (2020) with slight modification in extending the inoculum size to 20 % (v/v). Respective amount of inoculums (4, 8, 12, 16 to 20 % (v/v)) were inoculated into 50 ml of MSM-KL medium and incubated at pre-optimal condition (temperature: 50℃; incubation time:15 hours). Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.4 Effect of initial pH on crude LiP production

The *Bacillus licheniformis* was cultured in 50 ml MSM-KL medium at different pH ranging from pH 6 – 10 (Tuncer et al., 2004) to investigate the effect of initial pH on the LiP production after incubating at pre- optimal condition (temperature:

50℃; incubation time:15 hours; inoculum size: 16% (v/v)) under static condition. Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.5 Effect of glucose concentration on crude LiP production

The effect of glucose concentration on crude LiP production by *Bacillus licheniformis* was examined at glucose concentration of 0, 0.2, 0.4, 0.6 and 0.8% (w/v) in 50 ml MSM-KL medium under pre- optimal condition (temperature: 50 ℃; incubation time:15 hours; inoculum size: 16% (v/v); pH 7). Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.6 Effect of yeast extract on crude LiP production

To investigate the effect of yeast extract concentration on the crude LiP production by *Bacillus licheniformis* in 250 ml flask containing 50 ml MSM-KL medium, yeast extract concentration was varied from 0 % (w/v) to 0.8 % (w/v). The yeast extract concentration range was referred to study by Rekik et al. (2015) with slight modification to fit into the efficient LiP production (Rekik et al., 2015). The medium was incubated at pre- optimal condition (temperature: 50 ℃; incubation time:15 hours; inoculum size: 16% (v/v); pH 7; glucose concentration: 0.4% (w/v)). Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.7 Effect of lignin concentration on crude LiP production

Kraft lignin concentration was varied from 0 % (w/v) to 0.1 % (w/v) to determine the effect of lignin concentration on crude LiP production by *Bacillus licheniformis* in MSM-KL medium under pre- optimal condition (temperature: 50℃; incubation time:15 hours; inoculum size: 16% (v/v); pH 7; glucose concentration: 0.4%

(w/v); YE concentration: 0.2% (w/v)). There is very limited study on the effect of kraft lignin concentration on crude LiP production, the selected kraft lignin concentration range was referred to the kraft lignin concentration in the solid plate assay suggested by Abdul Rahman et al. (2013). Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.8 Effect of $CaCl_2$ on crude LiP activity

The effect of $CaCl_2$ concentration on crude LiP production through submerged fermentation of *Bacillus licheniformis* in 50 ml of MSM-KL medium was examined at varying concentration from 0.02 % (w/v) to 0.1 % (w/v) under pre-optimal conditions (temperature: 50℃; incubation time:15 hours; inoculum size: 8% (v/v); pH 7; glucose concentration: 0.4% (w/v); YE concentration: 0.2% (w/v); Lignin concentration: 0.08 % (w/v)). The $CaCl_2$ concentration range was referred to study by Chauhan (2019) with slight modification to fit into the efficient LiP production. Five millitre of culture was withdrawn, centrifuged (4000 rpm for 10 min, 4℃) and the crude LiP enzyme activity in the supernatant was determined using standard LiP enzyme assay stated in section 2.3.1.1.

3.8.9 Data Analysis

The result presented in LiP production are the mean value of three replicates, and the standard deviation were used to analyse experimental data. Statistical analysis was performed via One-way Analysis of Variance (ANOVA) using Microsoft Excel. The results were considered statistically significant for *P* values of less than or equal to 0.05.

3.8.10 FTIR analysis on degradability of *Bacillus licheniformis* on kraft lignin

Delignification process is about degrading or depolymerises the lignin structure. Thus, in order to evaluate the potential of *Bacillus licheniformis* CLMTCHB29 on lignin degradation, it is crucial to examine and identify the degradability of *Bacillus licheniformis* CLMTCHB29 on kraft lignin by investigating the structural change in

the kraft lignin content. The kraft lignin degradation by *Bacillus licheniformis* CLMTCHB29 was examined by culturing *Bacillus licheniformis* CLMTCHB29 under optimal nutritional and culture condition in MSM-KL medium and another uninoculated MSM-KL medium served as control. Each culture medium was harvested after 96 hours of incubation, the sample collected from inoculated and uninoculated medium were denoted as treated lignin and untreated lignin sample respectively. The collected samples were centrifuged, and the solid residue (lignin powder) settled at the bottom of centrifuge tube was collected and dried in oven at 90℃ until constant weight was reached. These dried lignin samples were used in the subsequent FTIR analysis to analyse its lignin structure. FTIR sample was prepared with potassium bromide (KBr) pellet method by mixing dried KBr with dried lignin powder in the weight ratio of 100:1. A thin KBr wafer was formed when the mixture was compressed at 10 t/cm^{-2} pressures for 1 min (Liu et al., 2014). Two types of samples (treated lignin powder, and untreated lignin powder) were prepared according to the method above. FTIR spectra were measured in the region of 4000 – 400-cm^{-1} by an Agilent Cary 630 Fourier Transform Infrared Spectroscopy using transmittance with a resolution of 1 cm^{-1}. Lignin is made up from guaiacyl, syringyl and lignin subunit. Thus, the transmittance value at wavenumber 1123-1110, 1219, 1268, 1426, 1460, 1515-1511 and 1615-1600 that were known closely related to aromatic bonding in guaiacyl, syringyl and lignin subunit was obtained from scanned FTIR spectra for both treated and untreated lignin sample. Nonetheless, the percentage of degradation of kraft lignin was determined using transmittance value at 1515- 1511 cm^{-1} as suggested by by Liu et al. (2014) (Eqn. 2) since the absorbance at this wave number was found to have high correlation with the C=C stretching of the aromatic ring in lignin. Moreover, this absorbance could not be influenced by another functional groups through overlapping effect (Pandey, 1999).

$$\%\ of\ degradation = \frac{T_1 - T_0}{T_0}\ x\ 100\% \qquad \textit{Equation 2}$$

T_1 = transmittance value at 1515-1511 cm^{-1} in treated lignin sample
T_0 = transmittance value at 1515-1511 cm^{-1} in non treated lignin sample Enzyme purification

3.9 Enzyme purification

The main purpose of LiP purification process was to obtain pure LiP enzyme for characterisation purpose. Characterisation study is to evaluate the feasibility and suitability of an enzyme for industrial application. The advantage of using purified LiP over crude LiP in characterisation study is to avoid interference from other enzymes or substrates that may be present in the crude enzyme. A 500 ml volume of 15 h old culture medium was fermented under the optimal condition that has been determined in the previous section. The culture medium was centrifuged for 20 min at 4,000 rpm in refrigerated centrifuge (Brand: Hettich; Model: Universal 320R) to remove microbial cells. The supernatant was collected, and vacuum filtered with 0.22µm filter for subsequent use in the following purification steps.

3.9.1 Ultrafiltration

The vacuum filtered crude enzyme supernatant (50 mL) was subjected to ultrafiltration with 10 kDa cut-off Ultracel regenerated cellulose membrane (Merck) in Amicon 8050 dead end stirred cell (50 mL) (Millipore). In the ultrafiltration step, 500 mL of culture supernatant concentrated at pressure of 60 psi and 100 rpm stirring speed under room temperature. The retentate was collected, pooled together, and stored at 4℃.

3.9.2 Gel filtration chromatography

The ultrafiltrated retentate (1 ml) was loaded onto Bio-gel P100 gel filtration chromatography (Bio-Rad), 20 cm x 1 cm equilibrated with 20 mM sodium phosphate buffer (pH 7). The column was eluted with 20 mM sodium phosphate buffer at 4 ml/h and 1 ml of fraction was collected in microcentrifuge tube.

The absorption spectrum at 280 nm and 409 nm for each fraction were measured with Perkin-Elmer spectrophotometer to determine the protein content and heme-protein content, respectively. The LiP enzyme activity in each fraction were determined using standard LiP enzyme assay mentioned in section 2.3.1.1. The protein concentration was determined by Bradford protein assay (Sigma) and measured using UV-Vis spectrophotometer at 595 nm. Bovine serum albumin (BSA) (Sigma) was

used as protein standard. Fractions which showed LiP positive activity were pooled and stored at 4℃ to be used in characterization study.

3.10 Enzyme characterisation study

Enzyme properties such as enzyme stability, mediator involved, potential redox of the enzyme and substrate specificity are important factors in determining the feasibility of application for industrial use. Thus, a series of study was carried out to determine the fundamental characteristics and function of the enzyme. Experimental studies including enzyme kinetics, pI, substrate specificity, effect of metal ions and inhibitor on LiP were designed to address the potential redox of enzyme and substrate and potential mediator. The effect of pH, and temperature studies were performed to examine the stability of the enzyme.

3.10.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The SDS-PAGE was carried out using Mini-PROTEAN tetra cell (Bio-Rad) to determine the molecular weight of purified LiP enzyme. The handcast gel, running buffer solution and assembly of Mini-PROTEAN tetra cell tank were prepared according to standard protocol provided by Bio-Rad. The protein samples (vacuum filtered crude enzyme, ultrafiltrated, purified LiP) were diluted with SDS reducing buffer with ratio of 1:2 and heat at 95℃ for 4 min prior to sample loading. 30 µl of samples (vacuum filtered crude enzyme, ultrafiltrated, purified LiP) and Chromatein prestained Protein ladder (Vivantis; PR0602) were loaded into the well. The proteins were separated by SDS-PAGE using 12% w/v resolving gel and 5% w/v stacking gel in Tris/glycine/SDS running buffer (pH 8.3). The SDS-PAGE was running under power condition at constant voltage 100 V for 1.5 hr.

Modified Coomassie Brilliant Blue (CBB) G-250 staining protocol (Kang et al., 2002) was employed to stain and visualise the band. The staining solution consists of 0.02 % (w/v) CBB G-250, 5% (w/v) aluminium sulfate octadecahydrate, 10 % (v/v) ethanol (96%) and 2 % (v/v) orthophosphoric acid (85%). The sensitivity of the staining solution is highly dependent on the order of dissolving the above chemicals. The aluminium sulfate was first dissolved in Milli-Q water and ethanol was then added

and homegenised. After addition of ethanol, CBB-G 250 was then added to the solution until it was completely dissolved and subsequently followed by phosphoric acid. The protein bands were stained with staining solution overnight at room temperature followed by destaining using Milli-Q water.

3.10.2 Isoelectric point of purified LiP

The isoelectric point of purified LiP enzyme was determined by measuring the zeta potential using Zetasizer Nano-ZSP (Malvern Panalytical) with the aid of autotitrator MPT-2 in adjusting the pH. The zeta potential of the enzymes was measured from pH 0.5 to 11. Isoelectric point is the pH value where the enzyme showed zero zeta potential. The zeta potential was measured using dynamic light scattering method whereby the solvent refractive index and laser light scattering were set at 1.335 and 173˚. This is the default setting for measuring the isoelectric point of protein in solution form (Schultz et al., 2008). One millimetre of purified LiP enzyme was loaded into disposable folded capillary cuvette cell and all zeta potential measurements were made at 25℃.

3.10.3 Kinetic study of purified LiP

The kinetic parameters of the purified LiP were determined at room temperature in the presence of varying concentrations of veratryl alcohol and H_2O_2. The LiP activity on H_2O_2 was assayed on 2.0 mM veratryl alcohol in citrate-phosphate buffer (pH 2.7) with H_2O_2 concentration ranged from 0.4 mM to 8.0 mM. Similarly, the LiP activity on veratryl alcohol was assayed using 0.8 mM H_2O_2 with varying veratryl alcohol concentration from 1.0 mM to 20 mM. The data obtained were used to construct the Lineweaver Burk plot and subsequently determine the Michaelis Menten constant (K_m) and maximum rates for both substrates (veratryl alcohol and H_2O_2).

3.10.4 Substrate specificity

Substrate specificity of the purified LiP (500 µl) was determined using veratryl alcohol (ε_{310} = 9300 M^{-1} cm^{-1}), guaiacol (ε_{465} = 12100 M^{-1} cm^{-1}), dimethoxyphenol (ε_{469} = 49600 M^{-1} cm^{-1}) (DMP), ABTS (ε_{420} = 36000 M^{-1} cm^{-1}), Azure B (ε_{651} = 22000

M^{-1} cm^{-1}) (Archibald, 1992), Malachite Green (ε_{617}= 8400 M^{-1} cm^{-1}) (Yu et al., 2014) and Phenol Red (ε_{564}= 30737 M^{-1} cm^{-1}) (Shrivastava et al., 2005). LiP activities against veratryl alcohol, guaiacol, DMP; ABTS; Azure B, Malachite Green and Phenol Red were assayed at final concentration 2 mM, 1 mM and 32 µm respectively (Rekik et al., 2019, Lueangjaroenkit et al., 2019, Yu et al., 2014).

3.10.5 Optimum pH and pH stability

The optimum pH of purified LiP (250 µl) was examined at pH 1.5, 2.0, 2.5, 2.7, 3.0 and 3.5. The LiP activity was measured using standard assay method by replacing the 100 mM citrate phosphate buffer (pH 2.7) with the following buffer systems: 50mM KCl-HCl buffer (pH 1.0-2.5) and 50 mM citrate phosphate (pH 2.5-4). The LiP activity at optimum pH was taken as 100%. The relative activity is denoted as below (Lueangjaroenkit et al., 2019) (Eqn 3).

$$Relative\ activity = \frac{LiP\ activity\ of\ sample}{LiP\ activity\ at\ optimum\ pH} x100\% \qquad Equation\ 3$$

pH stability of purified enzymes was measured at room temperature by incubating the 1.25 ml enzyme solution for 48 h in the following buffer system ranging from pH 3 to 9: 50mM KCl-HCl buffer (pH 1.0-2.5) and 50 mM citrate phosphate (pH 2.5- 4), 50 mM sodium acetate (pH 4-5) or 50 mM sodium phosphate (pH 6-8) or Tris-HCl buffer (pH9). 250 µl enzyme samples were withdrawn at specific time (0 min, 10 min, 1, 2, 4, 10, 16, 24 and 48 h) and LiP activity was determined using the standard LiP assay. LiP activity at 0 min for each sample was used as control (100%). The residual activity is defined as LiP activity at each specific time with respect to LiP activity at 0 min.

3.10.6 Optimum temperature and temperature stability

The optimum temperature of the purified LiP was examined from 25℃ to 80℃. The enzyme solution and mixture of assay (100 mM citrate phosphate buffer and veratryl alcohol) were incubated separately in Waterbath WNB14L (Memmert, Germany) at the desired temperature for 15 min. Five hundred microlitre enzyme solution was then added into mixture assay and reaction was initiated with H_2O_2 according to standard LiP assay. The LiP activity at optimum temperature was taken

as 100%. The relative activity is denoted as below (Lueangjaroenkit et al., 2019) (Eqn 4) .

$$Relative\ activity = \frac{LiP\ activity\ of\ sample}{LiP\ activity\ at\ optimum\ temperature} x100\% \qquad Equation\ 4$$

For temperature stability study, 3 ml enzyme solutions were incubated at temperature ranging from 60℃ to 70℃ for 24 h, 500 µl were withdrawn at specific time (1, 2, 4, 10, 16, 24 h) and LiP activity was measured using standard LiP assay. LiP activity at 0 min for each sample was used as control (100%). The residual activity is defined as LiP activity at each specific time with respect to LiP activity at 0 min.

3.10.7 Effect of metal ions on purified LiP

The effect of metal ions on purified LiP activity were investigated by adding final concentration of 1 mM of each metallic salt ($FeCl_2$, $FeCl_3$, $MgCl_2$, KCl, NaCl, $CaCl_2$, $MnCl_2$ and $NiCl_2$) to the reaction mixture. The concentration of metal ions was selected as suggested by Lueangjaroenkit et al. (2019). The enzyme sample containing 250 µl of purified LiP was incubated in 250 µl of 20 mM phosphate buffer (pH 7) containing respective metal ions at 30℃ for 30 min (Bouacem et al., 2018). LiP activities measured in the absence of metallic ions (replaced with ultrapure water) were taken as control (100%). The activity for samples containing metal ions were then assayed according to standard LiP assay. The relative LiP activity was defined as LiP activity of sample containing metal ions with respect to control sample (Eqn. 5).

$$Relative\ activity = \frac{LiP\ activity\ of\ sample\ containing\ metal\ ions}{LiP\ actvity\ of\ control\ sample}\ x\ 100\% \qquad Equation\ 5$$

3.10.8 Effect of inhibitors on purified LiP

The effect of inhibitors on LiP activity were determined by adding final concentration of 0.1 mM of each possible inhibitor (SDS, EDTA, Tween-80, NaN_3) to the reaction mixture. Two hundred fifty microlitre of purified LiP enzyme sample was incubated in 250 µl of 20 mM phosphate buffer (pH 7) containing inhibitors at 30℃ for 30 min. LiP activities measured in the absence of inhibitors (replaced with ultrapure

water) were taken as control (100%). The activity for samples containing inhibitors were then assayed according to standard LiP assay. The relative LiP activity was defined as LiP activity of sample containing inhibitors with respect to control sample (Eqn. 6).

$$Relative\ activity = \frac{LiP\ activity\ of\ sample\ containing\ metal\ ions}{LiP\ actvity\ of\ control\ sample}\ x\ 100\% \qquad Equation\ 6$$

Chapter 4 Isolation and Identification of Thermophilic Ligninolytic Bacteria

4.1 Screening and isolation of thermophilic bacteria

In the primary screening, MSM agar plate with kraft lignin as sole carbon was used to screen potential ligninolytic bacteria. In this screening, a total of 34 bacteria isolates were successfully isolated. Use of MSM-KL agar plate assay for selection of ligninolytic bacteria is viewed as one of the most suitable methods among the wide variety suggested by several authors (Falcon et al., 1995, López et al., 2006). This plate assay was used to eliminate the bacteria that could not feed on kraft lignin as the sole carbon source. Colonisation of these bacteria observed in MSM-KL without glucose indicated that the isolates were able to mineralise kraft lignin as the carbon source in the absence of glucose.

To study the ligninolytic potential independently from lignin utilization, these 34 bacteria were further screened with both MSM-KL containing YE and glucose; and Tryptic Soy agar (TSA) plate with lignin mimicking dye-Methylene Blue. Bandounas et al. (2011) suggested some bacteria may rely on specific nutrients such YE to fulfill the role of lignin degrader or dye decolourization ability. Hence, two types of MB agar were used to screen the potential ligninolytic bacteria. In both plate assay, 10 isolates showed growth but no decolourize zone for MB were observed in any of the plates. However, the growth of the isolates in the MSM-KL containing YE and glucose; and Tryptic Soy agar (TSA) plate with MB dye indicate MB could sustain the bacteria growth in the presence and absence of glucose respectively. Although polymeric dye plate is a direct and easily visualize solid plate assay used in screening potential ligninolytic bacteria, it is not necessarily a good measure of the ligninolytic potential of a bacteria isolate. The metabolic pathway in dye decolourization of bacteria is complex and some lignin degrading bacteria require additional nutrients for dye decolourization (Saratale et al., 2009). This is supported by supplementation of YE was reported to be essential in dye decolourization which suggested that *Bacillus* sp. LD003 may fulfill the role of lignin degrader in decolourise MB that has to rely on specific nutrients (Bandounas et al., 2011). In fact, several soil inhabiting ligninolytic

fungi also demonstrated low correlation between lignin-degradation activity and decolourisation of polymeric dyes (Falcon et al., 1995, Pasti and Crawford, 1991). Similar observation was also reported on *Streptomyces psammoticus* that is capable in producing ligninolytic enzymes, but possesses no decolourisation ability on dyes, such as Poly R and RBBR (Niladevi and Prema, 2005). *Pandoraea norimbergensis* LD 001 had been reported to degrade lignin monomers with no decolourisation ability on MB (Bandounas et al., 2011).

Apart from polymeric dye plate assay, incorporation of guaiacol in solid screening media was selective in isolating ligninolytic bacteria since guaiacol can be degraded by ligninolytic enzymes including LiP, MnP and Lac. Thus, these isolates were further screened with MSM assay plate supplemented with guaiacol as the sole carbon source. Among 34 isolates, guaiacol could sustain the growth for ten isolates that are listed in table 4.1 and no clear zone was observed in those plate even after one week of incubation. Similarly, Niladevi and Prema (2005) reported that two mangrove actinomycetes isolate NJP 46 and NJP 47 with no capability in decolourising guaiacol solid plate were found to produce ligninolytic enzymes in the liquid production medium. Compared with those in previous studies, the ten isolates in the present study exhibited better cell growth when using lignin, dye, and lignin monomer as the sole carbon source. The growth superiority of these isolates indicated their potential in mineralising lignin like structure compound (Xu et al., 2018). No decolourisation was observed. This may be due low specificity of the enzymes towards guaiacol or low production of the ligninoltic enzyme on solid agar assay. In this context, a more precise and quantitative spectrophotometric enzyme assay could resolve the above issue (Niladevi and Prema, 2005).

Table 4.1 The growth of bacteria using three different agar plate assay

Isolates	MSM-KL+ MB[a]	TSA+MB[b]	Guaiacol[c]
CLMT 5	+	++	+
CLMT 18	+	+++	+
CLMT 19	+	++	+
CLMT 20	++	+++	+++
CLMT 21	++	+++	++
CLMT 22	++	+++	++
CLMT 23	+	++	+
CLMT 28	+	++	+
CLMT 29	+	++	+
CLMT 34	+	++	+

[a]MSM-KL + 25 mg/L MB dye + 2.5 g/L Yeast extract + 2 g/L glucose
[b]TSA + 25 mg/L MB dye
[c]MSM-KL + 1 mM guaiacol
+++; very fast growth (colonies visible after 12 hours)
++; fast growth (colonies visible after 18 hours)
+; slow growth (Visible colonies after 24 hours)

4.2 Ligninolytic Enzyme profiles

To investigate the ligninolytic production from the potential isolates, the ligninolytic enzyme profiling was undertaken to screen ligninolytic enzyme producer and determine the most potent bacteria isolates among the tested isolates for further study. Ten isolates were cultured in respective MSM-KL medium supplemented with glucose and yeast extract under submerged fermentation at 50℃, pH 7 for a period of 120 h. The ligninolytic enzymes activity in the cell free supernatant were determined at every 24 hours. The LiP, MnP and laccase's enzyme profile for each culture were determined and plotted in Figure 4.1, 4.2 and 4.3 respectively. All tested isolates showed positive result in producing primary ligninolytic enzyme simultaneously with LiP as the predominant enzyme followed by MnP and almost insignificant amount of laccase. Isolate CLMT 29 attained the highest LiP enzyme activity (8.7672 U/L) among all the tested isolates. Generally, the MnP and Lac activity were ranged from 0.4 -1.9 U/L and 0.4 - 0.8 U/L respectively. In term of the enzyme profile, most of the isolates achieve maximum LiP and MnP production at 24 h. Decrease and increase of LiP and MnP enzyme activity were observed before and after 48 h and 72 h respectively in most of the isolates. On the contrary, the induction of laccase was only observed after 24 h and continually to increase towards the end of the fermentation process. This

might be due to the depletion of nitrogen source towards the end of fermentation process.

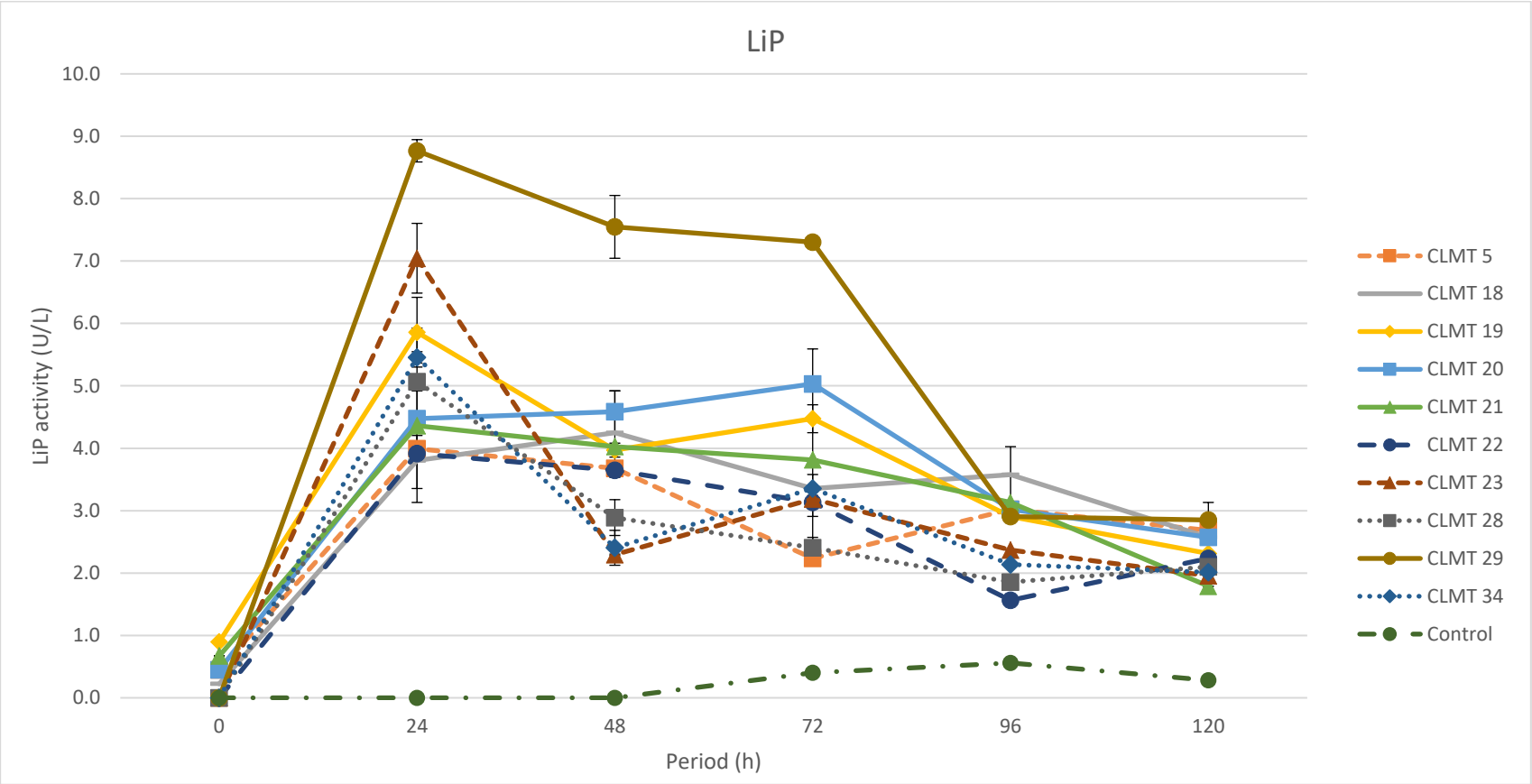


Figure 4.1 Time course of LiP production by ten isolates for 120 h at 50℃ under submerged fermentation using MSM-KL (pH 7) supplemented with 10 g/L glucose, 1.0 g/L yeast extract. Uninoculated MSM-KL flask served as control. Each point represent the mean value with the standard error of three replicate.

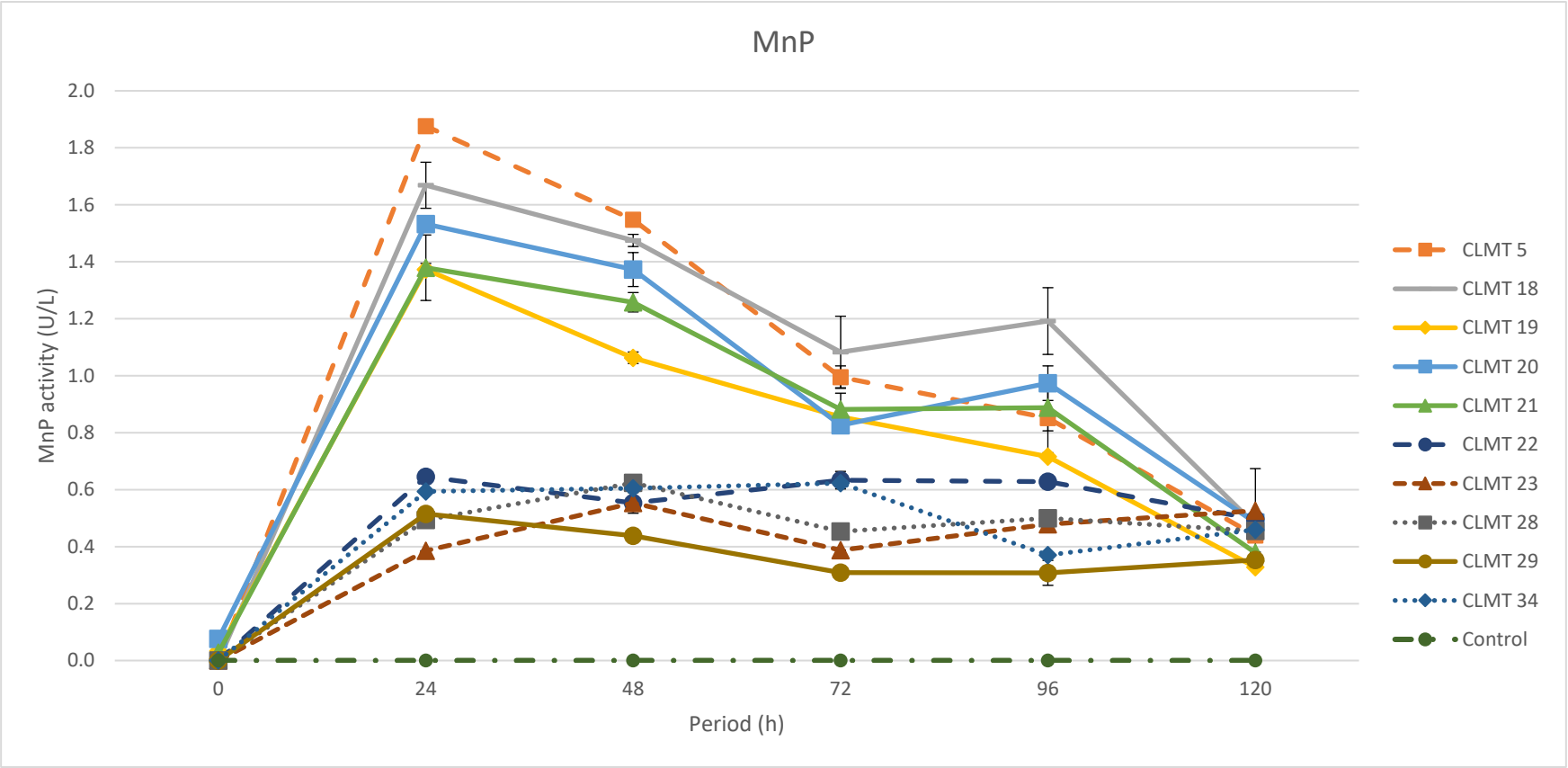


Figure 4.2 Time course of MnP production by ten isolates for 120 h at 50℃ under submerged fermentation using MSM-KL (pH 7) supplemented with 10 g/L glucose, 1.0 g/L yeast extract. Uninoculated MSM-KL flask served as control. Each point represent the mean value with the standard error of three replicate

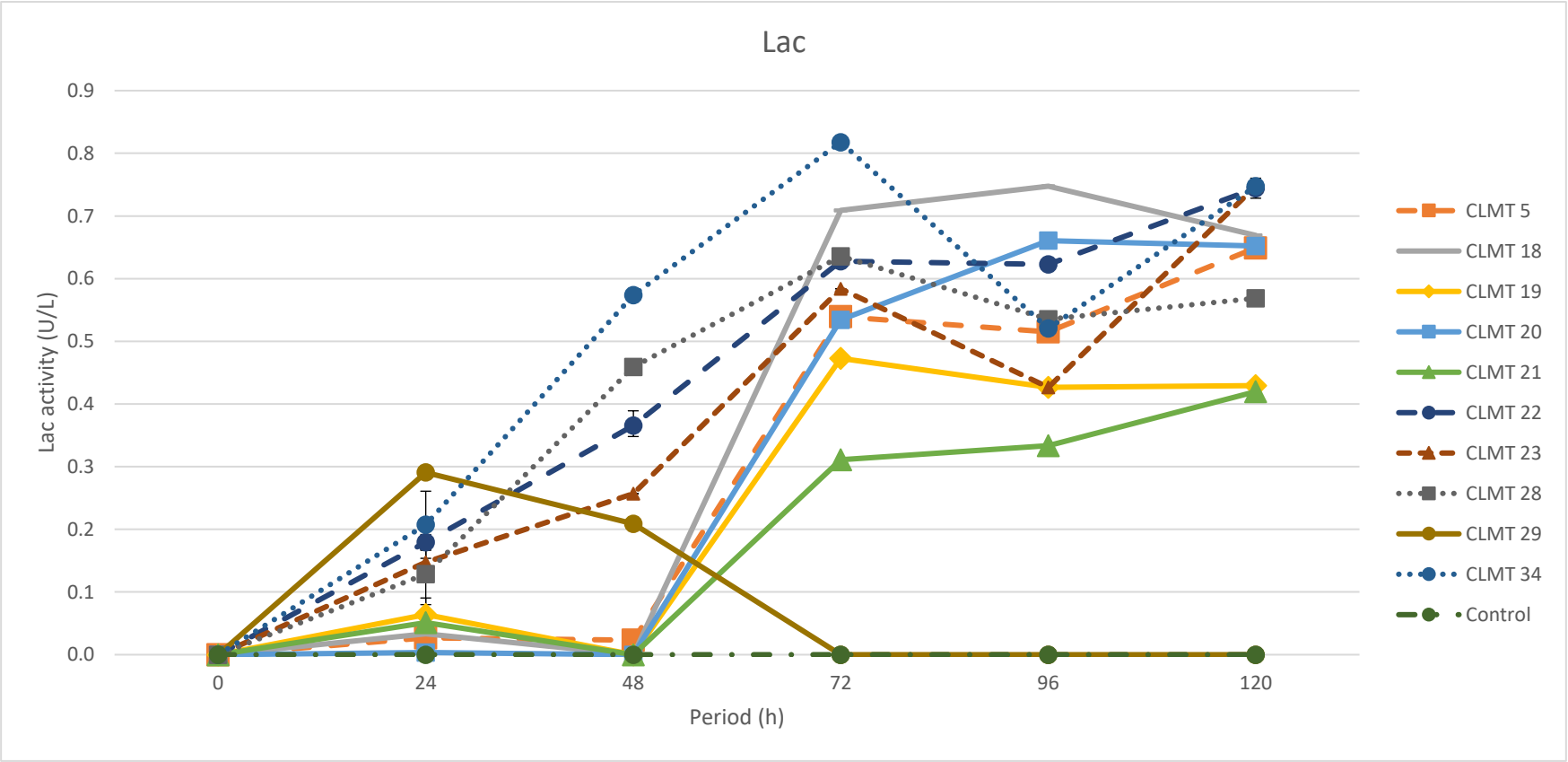


Figure 4.3 Time course of Lac production by ten isolates for 120 h at 50℃ under submerged fermentation using MSM-KL (pH 7) supplemented with 10 g/L glucose, 1.0 g/L yeast extract. Uninoculated MSM-KL flask served as control. Each point represent the mean value with the standard error of three replicate.

Capability of *Bacillus licheniformis* in producing three primary ligninolytic enzyme is rarely reported. Most of the bacteria were reported to produce only two types of ligninolytic enzymes in the combination of LiP with laccase or MnP with laccase (Chen et al., 2012b, Shi et al., 2013b, Chen et al., 2012a, Buraimoh et al., 2015). A thermophilic rumen ligninolytic bacterium RUFR60 was documented capable of secreting Lac, MnP but no LiP was detected (Seesatat et al., 2021). In contrary, *Bacillus cereus* used to detoxify paper mill effluent was found capable of secreting LiP, MnP and Lac (Kumar et al., 2022). Nevertheless, Yang et al. (2017) also revealed several lignin degrading bacteria isolated from Qinling were capable in producing LiP, MnP and laccase. The maximum production the LiP and MnP at 24 h is considered relatively quick and the enzyme profile is quite similar with *Pseudomonas* sp., *Ochrobactrum* sp. that showed maximum production within short period of time at 24 hours and gradually decreased until termination of fermentation process. In contrast, laccase activity increased steadily until 72 hours and maintained its maximum production towards the end of fermentation (Yang et al., 2017). Similar enzyme profile was also reported in *B. subtilis* and *Klebsiella pneumonia*. The phenomena of peroxidase enzyme production being active at the early stage could be due to large lignin polymer molecules that are not able to traverse into the cell membrane in the initial stage. Thus, extracellular enzymes such as peroxidase enzymes are secreted externally to degrade lignin polymer compounds to smaller fragment during primary metabolism (Yadav and Chandra, 2015).

The phenomenon of declination and reclination of LiP and MnP enzyme production observed in this study is in accordance with the study by Yang et al (2017) whereby the enzyme profile of LiP, MnP and Lac for genus *Pseudomonas, Raoultella, Ochrobactrum* and *Acinetoobacter* showed fluctuation in its enzyme activity throughout the fermentation process. This may be attributed to the effect of high concentration of toxic lignin degradation by-products cause inhibition on bacteria growth or LiP and MnP enzyme activity. Variety of phenolic compounds or free hydroxyl phenolic structures are released during the biodegradation of lignin. Phenol, guaiacol, 2,6-DMP, vanillyl alcohol, vanillin, vanillic acid, coniferyl alcohol, coniferyl aldehyde and ferulic acid are the common by-products that was known to inhibit bacteria growth and affect the LiP and MnP enzyme production throughout the

fermentation process (Li and Wilkins, 2021, Pham et al., 2014). Study by Dietrich et al. (2013) showed that inhibition was observed in growth of *Bacillus*, *Pseudomonas* at 0.25g/L of coumaric and ferulic acid which subsequently reduce the enzyme production. The inhibition effect of coumaric and ferulic acid on microorganism vary considerably in their tolerance of, and their ability to catabolize phenolic compounds (Dietrich et al., 2013). However, the relationship between *Bacillus licheniformis*'s growth, enzyme activity and fermentation by-products in time course are yet to be explored. Further understanding on the types and effect of by-products on enzyme activity and growth in time course is required since enzyme production is a crucial aspect in any lignin degradation application.

The selected 10 isolates were gram stained and viewed under microscope to determine their morphology. Table 4.2 summarises the morphology and size of the bacteria cells under microscopic view. All isolates are gram positive with rod-shaped except for isolate CLMT 22 and CLMT 34 which are negative gram-stained bacteria. The microscopic view of Gram-stained bacteria isolates was attached in Appendix A.

Table 4.2 Morphology and size of the ten isolates

Isolates	**Morphology**	**size (μm) length, width**	**Gram staining**	**Colour of colonies**
CLMT 5	Rod shape	3.00, 0.67	+ve	Yellow
CLMT 18	Rod shape	2.33, 0.83	+ve	Yellow
CLMT 19	Rod shape	2.33, 0.67	+ve	Off-white
CLMT 20	Rod shape	2.00, 0.67	+ve	Yellow
CLMT 21	Rod shape	2.00, 0.50	+ve	Yellow
CLMT 22	Rod shape	2.67, 0.67	-ve	Yellow
CLMT 23	Rod shape	2.00, 0.83	+ve	Brown
CLMT 28	Rod shape	3.67, 0.50	+ve	Brown
CLMT 29	Rod shape	2.33, 0.83	+ve	Off-white
CLMT 34	Rod shape	2.67, 0.50	-ve	Off-white

*Colour of the colonies was observed from TSA plate

In the current study, LiP was found to be the predominant ligninolytic enzyme and comparatively, it is least studied enzyme from lignin degrading bacteria. Moreover, given its unique ability in degrading non-phenolic compounds that are not readily oxidised by MnP and Lac, it is worth exploring further. Extensive studies have shown

LiP activity in ligninolytic fungi. *Phanerochaete chrysosporium* are the typical mesophilic LiP fungi producer. Apart from that, Tomšovský and Homolka (2003) and Mohamad Ikubar et al. (2018) also reported LiP activity of 390 µkat/L, 310 µkat/L, 20 µkat/L and 222 U/g from *Trametes versicolor* DV14, *Trametes hirsuta* DH3, *Trametes ochracea* DZ2 and *Trichoderma virens* respectively (Tomšovský and Homolka, 2003, Mohamad Ikubar et al., 2018). Diverse marine fungi such as *Mucor racemosus* CBMAI 847 also demonstrated LiP activity of 75376.34 U/L (Bonugli-Santos et al., 2010). However, it was revealed that production of LiP from fungi face several challenges during cultivation at upscale application. LiP in its native system sensitives to shear force and high temperature. Genetic modification strategy was employed to increase the yield of LiP (Liu et al., 2021a, Majeke et al., 2020, Majeke et al., 2021). Maximum volumetric activity of 4487.4 U/L was reported in expression of LiP using *Pichia pastoris* strain using bioreactor. LiP originated from thermophilic *Thermothelomyces thermophilus* was produced at 37 ℃ with activity of 182 mU/L using a recombinant *Aspergillus nidulans* strain using corn steep liquor. Apart from that, ancestral mutation strategy was shown to be effective in enhancing the thermal stability of a recombinant LiP from *Phanerochaete chrysosporium* strain UAMH 3641. The enzymatic activity of ancestral mutant enzyme m10 was significantly enhanced by 69% after incubating at 37℃ for 10 min compared to wild type LiP. Moreover, ancestral mutant m10 LiP showed elevated optimum temperature at 40 ℃ with increasing specific activity while wild type LiP only active at temperature <30℃ (Semba et al., 2015). Although genetic engineering strategy was found significantly increase the thermal stability of fungal LiP, there are rarely fungi LiP showed great stability at elevated temperature above 45℃. The LiP activity in CLMT 29 is relatively lower than those reported in mesophilic bacteria such as *Burkholderia* sp. H1, genera *Streptomyces* and *Nocardia* (Table 4.3) (Yang et al., 2017, Le Roes-Hill et al., 2011) but much lower than *Bacillus amyloliquefaciens* SL-7, *Bacillus* FALADE-1 and *Streptomyces griseosproreus* SN9. Although CLMT 29 produces lower titres of LiP, the production of LiP at 50 ℃ indicates machinery of LiP synthesis from CLMT 29 is capable of functioning even at elevated temperature that is not observed in most ligninolytic fungi and bacteria. In recent study, Niu et al. (2021) reported *Brevibacillus thermoruber* also capable of secreting LiP at elevated temperature (55℃) attaining activity of 984.51 U/L and lignin degradation of 81.97%. The high lignin degradation efficiency indicate bacteria LiP probably involve in lignin degradation process and

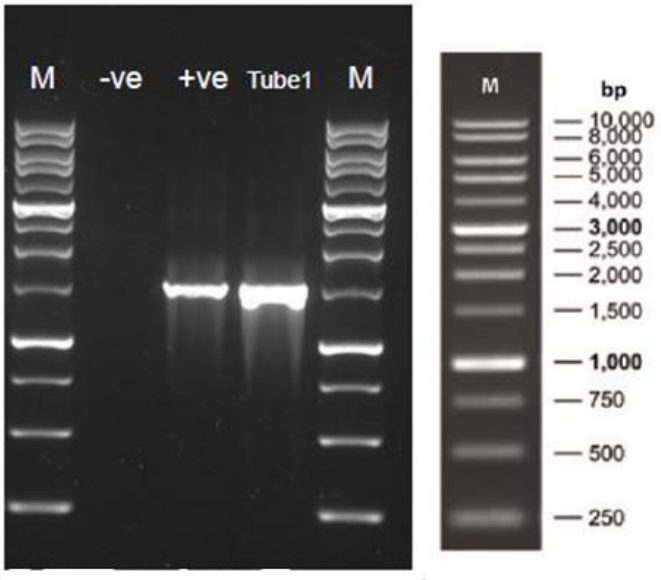
possessed similar degradation efficiency as what the fungi could offer (Niu et al., 2021). Although CLMT 29 produces lower titers of LiP but the production of LiP at 50 ℃ indicate machinery of LiP synthesis from CLMT 29 are capable of functioning even at elevated temperature that most ligninolytic fungi and bacteria could not offer. Thus, it is worth to discover and study potential of CLMT 29 in secreting thermostable LiP that may be responsible for lignin degradation. Hence, isolate CLMT 29 which attained highest titers of LiP (8.7672 U/L) among the tested isolates was chosen for further examination and identification.

Table 4.3 Comparison of LiP activity of Bacillus licheniformis CMLMTCHB29 with previous studies

Isolates	Temperature (℃)	LiP activities (U/L)	Reference
***Bacillus licheniformis* CLMTCHB29**	50	8.7673	Current study
***Streptomyces* MV 24**	30	53	(Le Roes-Hill et al., 2011)
***Nocardia* MV 12**		17	
***Nocardia* MV 20**		6	
***Burkholderia* sp. H1**	37	5.677	(Yang et al., 2017)
Bacillus licheniformis	37	180	(Azizi-Shotorkhoft et al., 2016)
O. intermedium		210	
M. paludicola		101	
***Endomelanconiopsis* sp.**	30	345.36 x 10^3	(Nayana et al., 2020)
***Bacillus amyloliquefaciens* SL-7**	37	422.68	(Mei et al., 2020)
***Bacillus* FALADE-1**	30	8870	(Falade et al., 2019d)
Thermothelomyces thermophilus* recombinant *Aspergillus	37	1.645	(Liu et al., 2021a)
***Streptomyces griseosproreus* SN9**	45	172	(Rekik et al., 2015)

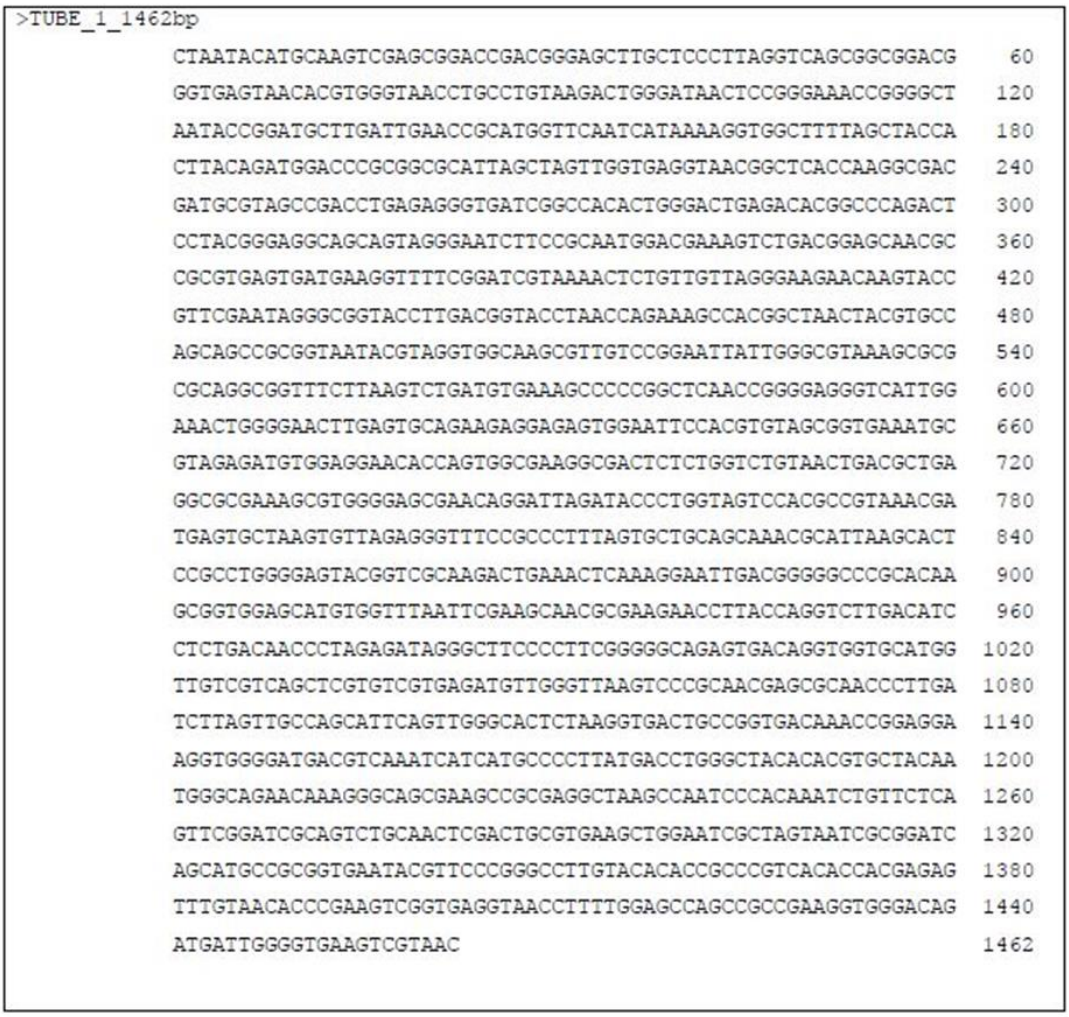
4.3 Bacteria identification

The PCR product obtained from the sequencing of the bacterial genome of the isolate CLMT 29 (Figure 4.4) is approximately 1462 bp length with its sequence shown in Figure 4.5. Comparison of the obtained sequence of the 16S rRNA gene with the database in GenBank using BLASTn tool indicated that the isolate is classified as *Bacillus* species. As shown in table 4.4, CLMT 29 showed 100% identity with *Bacillus licheniformis* DSM 13 (Accession number: NR 118996.1) and *Bacillus licheniformis* BCRC 11702 (Accession number: NR 116023.1). Apart from that, CLMT 29 was also closely related to *B. licheniformis* ATCC 14580 (Accession number: NR 074293.1), *B. licheniformis* NBRC 12200 (Accession number: NR 113588.1), *B. sonorensis* NBRC 101234 (Accession number: NR 113993.1) and *B. aerius* 24K (Accession number: NR 042338.1) with 99.0% similarity. Those sequences were imported into the MEGA software and multiple nucleotide sequence alignment was performed. A phylogenetic tree was then constructed using neighbour-joining method (Figure 4.6), and the findings further confirmed that the strain CLMT 29 was closely related to those of the *Bacillus licheniformis* strains. Thus, it was deposited in GenBank database named as *Bacillus licheniformis* strain CLMTCHB29 (Accession number: MH197076).



-ve: PCR negative control (water is used as DNA template)
+ve: Positive control (DNA extracted from E. coli is used as template)
Tube1: DNA extracted from isolate CLMT 29
M: Agarose gel electrophoresis marker

Figure 4.4 PCR product extracted from isolate CLMT 29



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>TUBE_1_1462bp
           CTAATACATGCAAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACG    60
           GGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCT   120
           AATACCGGATGCTTGATTGAACCGCATGGTTCAATCATAAAAGGTGGCTTTTAGCTACCA   180
           CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGAC   240
           GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACT   300
           CCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGC   360
           CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACC   420
           GTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCC   480
           AGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG   540
           CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG   600
           AAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGC   660
           GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGA   720
           GGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA   780
           TGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACT   840
           CCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA   900
           GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATC   960
           CTCTGACAACCCTAGAGATAGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGG  1020
           TTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGA  1080
           TCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGA  1140
           AGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAA  1200
           TGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTCA  1260
           GTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATC  1320
           AGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAG  1380
           TTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGAGCCAGCCGCCGAAGGTGGGACAG  1440
           ATGATTGGGGTGAAGTCGTAAC                                        1462
```

Figure 4.5 16S rRNA gene sequence for isolate CLMT 29

Table 4.4 Identification of closely related strain to isolate CLMT 29 based on 16S rRNA gene sequence using BLASTn tool

Bacteria	Accession number	Identity (%)
***Bacillus licheniformis* strain DSM_13**	NR 118996.1	100
***Bacillus licheniformis* strain ATCC 14580**	NR 074293.1	99.0
***Bacillus licheniformis* strain BCRC 11702**	NR 116023.1	100
***Bacillus licheniformis* strain NBRC 12200**	NR 113588.1	99.0
***Bacillus sonorensis* strain NBRC 101234**	NR 113993.1	99.0
***Bacillus aerius* strain 24K**	NR 042338.1	99.0

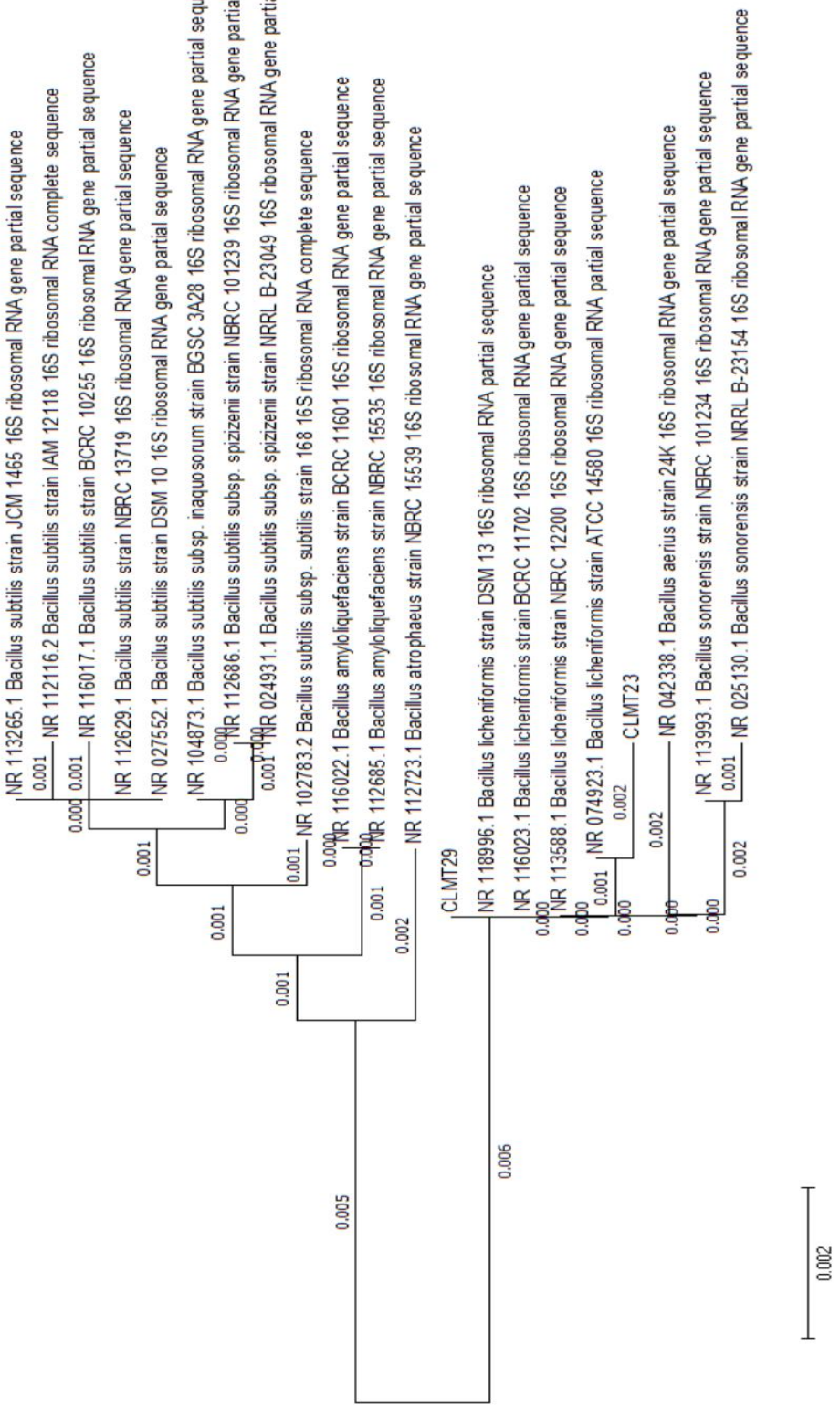

Figure 4.6 Neighbour joining Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic position of isolate CLMT 29 and related species with bootstrap value of 1000 replication

The isolated *Bacillus licheniformis* CLMTCHB29 exhibited different decolourization ability compared to those conventional lignin degrading fungi in solid plate assay method. Thus, the screening agar plate assay method that was initiated for lignin degrading fungi may not necessarily be suitable for lignin degrading bacteria. The studied isolate was able to produce three main ligninolytic enzymes namely LiP, MnP and Lac whereby LiP is the predominant enzyme.

Chapter 5 Production and optimal activity of LiP from Bacillus licheniformis

In this chapter, it presents about determining the efficient range of parameters for LiP production from *Bacillus licheniformis* CLMTCHB29. One factor at a time was employed to determine range of culture and nutritional factors that might impact the level of LiP produced and enhance the LiP activity. There were 9 parameters examined including fermentation configuration, incubation time, temperature, inoculum size, initial pH, glucose concentration, yeast extract concentration, lignin concentration and $CaCl_2$ concentration. Lastly, the kraft lignin degradation was carried out to examine the effectiveness of *Bacillus licheniformis* CLMTCHB29 in degrading kraft lignin. FTIR analysis was performed to analyse the change in lignin structure after the fermentation process to access its potential use in lignin degradability.

5.1 Cultivation of *Bacillus licheniformis* CLMTCHB29 using SSF and SmF

Production of LiP by *Bacillus licheniformis* was evaluated under both SSF and SmF. *Bacillus licheniformis* was grown in the MSM-KL medium (pH 7) at 50℃ under static condition for 96 h. Figure 5.1 presents the LiP activity of *Bacillus licheniformis* at every 24 h for both SSF and SmF. The results revealed that SmF was favoured in LiP production and no noticeable amount of LiP produced by *Bacillus licheniformis* was detected in SSF throughout the fermentation process. According to figure 5.1, *B. licheniformis* under SmF shows maximum LiP activity (10.16 U/L) at 24 h culture and activity starts to decline on the next 48 h of incubation. After 72 h of cultivation, LiP gradually decreases at a slower rate by attaining LiP production at about 3.5 U/L.

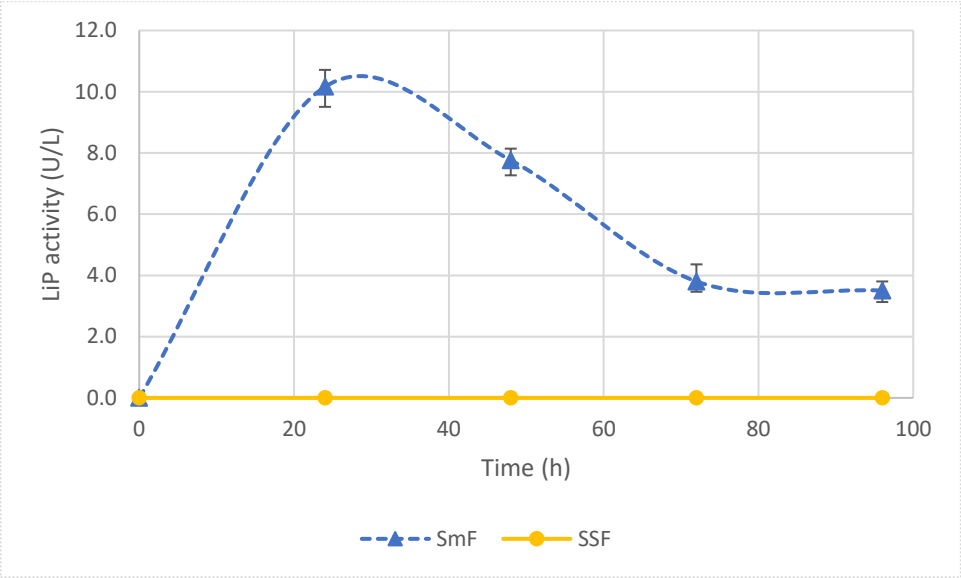

Figure 5.1 Time course of LiP production by B. licheniformis for 96 h at 50℃ under SmF and SSF cultivation using MSM-KL (pH 7) medium statically. Each point represents the mean result of three replicate with error bar indicating the maximum deviation from the mean value.

In contrast, Fenice et al. (2003) reported the presence MnP and laccase in both SmF and SSF of *Panus tigrinus* (Fenice et al., 2003). Similarly, Elisashvili et al. (2008) evaluated the productivity of laccase and MnP enzyme activity of *Pleurotus* sp. strains in submerged and SSF of various lignocellulose materials. MnP and Laccase were detected both SmF and SSF using tree leave. However, result revealed SSF is favourable in MnP and laccase production, whereas SmF decrease or inhibits these enzyme productions by *P. ostreatu*s 2191 and strain *P. osreatus* 2175. On the other hand, SmF appeared to be better technique in producing higher level of laccase when similar *Pleutorus* sp. strains were fermented in mandarin peels (Elisashvili et al., 2008). Similarly, Stajić et al., 2006 also suggested SmF was favourable in producing laccase from *P. eryngii* fermented in dry ground mandarine peels (Stajić et al., 2006). Nevertheless, the reason of *Bacillus licheniformis* CLMTCHB29 not producing under SSF may not due to the fermentation technique itself. As suggested by Kim et al. (2016), wide difference between the level of enzyme production under different fermentation technique of various lignocellulose could be attributed to the environmental condition (moisture content, aeration) and nature of the microorganism that affect the metabolic difference on the microorganism. In fact, it is proven that manipulation of cultivation method trigger the required alteration in growth morphology, growth kinetics, physiology, and chemical diversities such as production of secondary metabolites, enzymes (Kim et al., 2016). Since there is limited study on

LiP production by bacteria via SSF, a detailed nutritional and physical condition study maybe considered in the future to evaluate the potential of LiP production by *Bacillus licheniformis* via SSF.

5.2 One factor at a time approach for LiP production

A balanced fermentation medium is critical for good enzyme yield. The MSM-KL medium used for initial production of LiP by *Bacillus licheniformis* has to be modified for enhanced activity of LiP. Thus, the present study was designed to determine range of culture and nutritional factors that might impact the level of LiP produced and significantly enhance the LiP activity. This could provide a basic understanding on the nutritional and living requirement for this newly isolated thermophilic strain from EFB.

5.2.1 Effect of incubation time on LiP production

Bacillus licheniformis CLMTCHB29 was cultured in 50 ml of MSM-KL medium supplemented with 1.0 % (w/v) of glucose and 0.25 % (w/v) of yeast extract with 4% (v/v) inoculum. The medium was grown under 50℃ in static condition for 25 hours with initial pH 7. Figure 5.2. illustrates the time course of LiP production and growth by *Bacillus licheniformis* strain CLMTCHB29 for 25 h. It was observed that the LiP activity started after a 5-h lag phase, increased significantly during the growth of the organism, and attained its optimum peroxidase activity at 15 h (late logarithmic growth phase) with LiP activity of 10.28 U/L. The LiP activity started to decline at stationary growth.

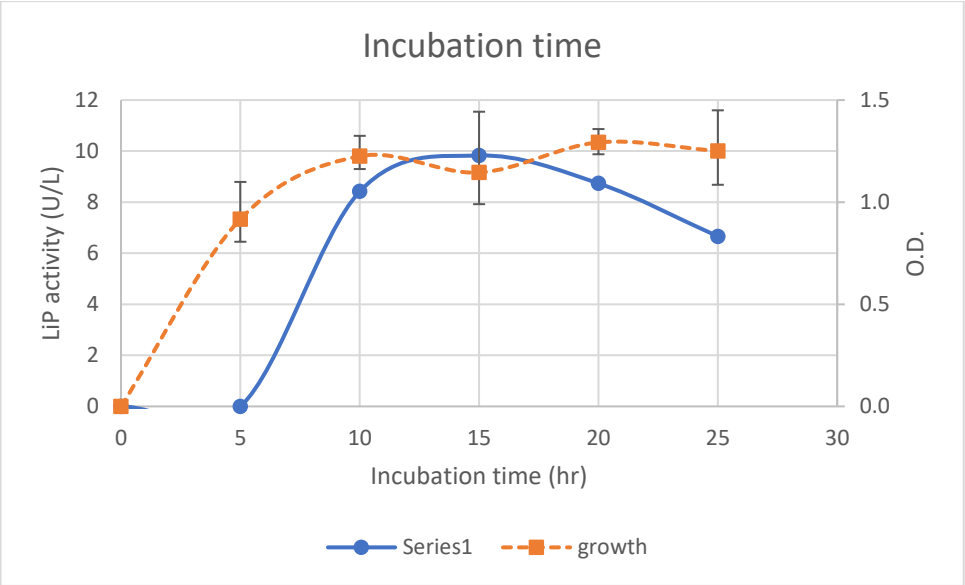

Figure 5.2 Effect of incubation time on LiP production by B. licheniformis for 25 h at 50℃ under SmF cultivation using MSM-KL (pH 7) medium supplemented with 1 (w/v)% glucose, 0.25 (w/v)% yeast extract, 2 (v/v)% inoculum, 0.05 (w/v)% KL, 0.05 (w/v)% $CaCl_2$.

The above finding agrees with study by Haq and Raj (2018) and Yang et al. (2017) that showed maximum LiP activity with shorter incubation time at 12 h and 24 h by *Pseudomonas putida F1* strain F1and *Serratia liquefacies* respectively (Haq and Raj, 2018). Apart from that, bacteria such as *Brevbacillus agri* AN-3 (Shah et al., 2018), *Streptomyces* sp. K37 and *Streptomyces* UAH30 reported a relatively long optimal incubation time at 72 h (Nour El-Dein et al., 2014, Rob et al., 1997). Nonetheless, *S. griseosporeus* and *Nonomuraea grerenzanensis* showed prolonged optimal incubation time at about 5 days and 20 days of cultivation. The variation in incubation time could be due to nature of the bacteria growth, fermentation medium and inoculum size. Nour El-Dein et al. (2014) and Falade et al. (2019b) suggested that decrease in level of peroxidase after optimal period could be due to denaturation and proteolysis activity or depletion of nutrients. Based on Figure 5.2, the continuous secretion of LiP is linked with bacteria growth. Thus, this indicated that production of LiP by bacteria was growth associated. Most of the previous study showed that peroxidase production by bacteria coincided with the active stage of growth. This included *E. adhaerens* NWODO_1 and *R. ornithinolytica* OKOH-1 except for *S. viridosprorus* T7A and *S. badius* 252 that are non-growth dependent where maximum peroxidase production was noted at the late stage of growth. (Lodha et al., 1991, Adhi et al., 1989),

5.2.2 Effect of temperature on LiP production

Figure 5.3 illustrate the effect of temperature on LiP production by *B. licheniformis* using MSM-KL medium at varying temperature from 45- 55°C for 15 h. The results in Figure 5.3 revealed that the *Bacillus Licheniformis* displayed highest LiP activity at 50℃. At incubation temperature lower than 50°C, extracellular LiP activity was noted to decrease 25% relative to maximal activity yields and temperature above 53°C did not favour LiP enzyme production. Similarly, LiP produced by *Brevbacillus agri* AN-3 and *S. griseosporeus* strain SN-9 showed optimum temperature at 50℃ and 45℃ respectively (Shah et al., 2018, Rekik et al., 2015). In contrast, previous study on other bacteria such as *Bacillus* sp. FALADE-1, *Bacillus* sp., *Bacillus subtilis* showed active production of LiP occurred at mesophilic temperature ranging from 30-37℃ (Falade et al., 2019d, Rajkumar et al., 2013, Rao and Kavya, 2014). To date, this is the first report on the thermotolerant LiP by *Bacillus licheniformis*.

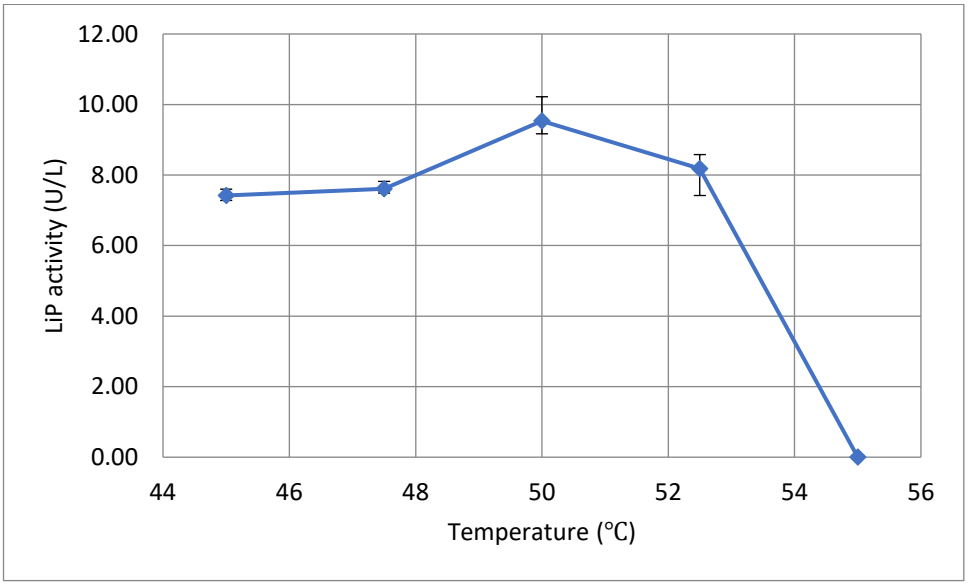

Figure 5.3 Effect of temperature on LiP production by B. licheniformis for 15 h under SmF cultivation using MSM-KL (pH 7) medium supplemented with 1% (w/v) glucose, 0.25 % (w/v) yeast extract, 2% (v/v) inoculum, 0.05% (w/v) KL, 0.05% (w/v) $CaCl_2$.

The narrow working temperature range was in accordance with isolate *Streptomyces* sp. K37 that showed no enzyme activity at 60°C, but *Stenotrophomonas* sp. CFB-09 still exhibited enzyme activity up to 80°C (Nour El-Dein et al., 2014, Olajuyigbe et al., 2018). This suggest that the working temperature range induced different level of enzyme actvity. The decline in enzyme yield below and above

optimum temperature might be attributed to reduction in metabolic activities of bacteria which consequently led to decrease in growth and enzyme formation. Increasing incubation temperature above 50°C could have also led to gradual inactivation of ligninolytic enzyme activity (Olajuyigbe et al., 2018, Falade et al., 2019b).

5.2.3 Effect of inoculum size on LiP production

The effect of inoculum size on LiP production by *B. licheniformis* was examined by inoculating a 10 h old inoculum at varying concentration from 4% (v/v) to 20% (v/v) into MSM-KL medium and incubated for 15 h for LiP production and result was presented in Figure 5.4. At inoculum size of 4% (v/v) and 8% (v/v), enzyme activity was minimum (64.03% of maximum yield) but as the inoculum level increased, the enzyme activity was also increased. At inoculum size of 18% (v/v), LiP activity increased by about 20% (6.64 U/L of LiP) and maximum yield was observed at 8% (v/v) of inoculum size. Further increased level of inoculum did not show appreciable changes on LiP activity.

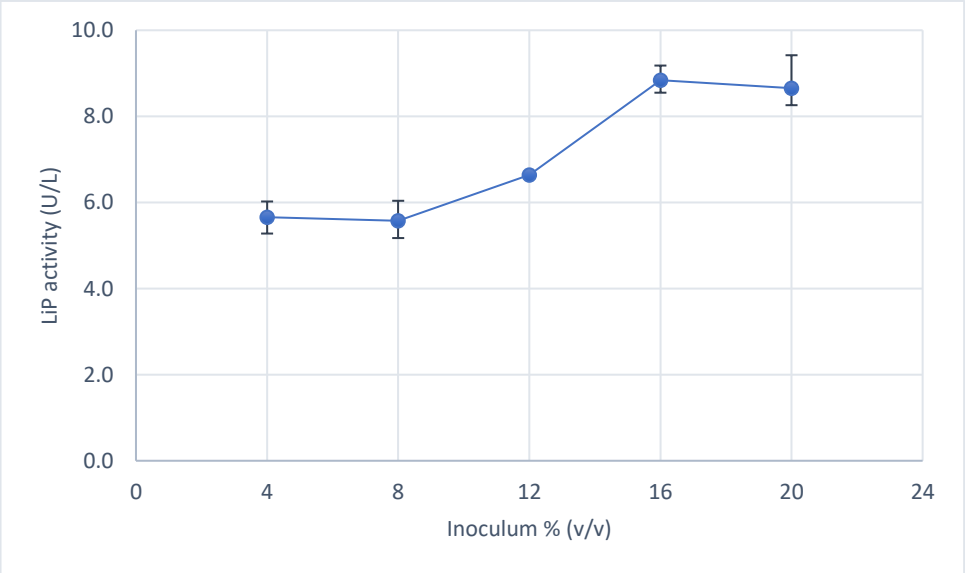

Figure 5.4 Effect of temperature on LiP production by B. licheniformis for 15 h at 50°C under SmF cultivation using MSM-KL (pH 7) medium supplemented with 1% (w/v) glucose, 0.25 % (w/v) yeast extract, 0.05% (w/v) KL, 0.05% (w/v) $CaCl_2$.

In recent study, a lower inoculum size of 2% (v/v) was found to be optimum for activity of LiP by psychrotrophic *Arthrobacter* sp. C2 and slight decrement was

observed at inoculum level above 2% (v/v) (Jiang et al., 2020). Similarly, study on LiP production by *Endomelanconiopsi*s sp. demonstrated maximum LiP activity of about 100 U/mL submerged fermentation at inoculum concentration of 3 mycelial plugs in modified Tien and Kirk medium (Nayana et al., 2020). Low LiP activity was observed with further increase in mycelial plugs, Nayana et al. (2020) reported 70% reduction or almost total lost in LiP activity at inoculum concentration of 4 and 5 mycelial plugs respectively. Similarly, Hariharan and Nambisan (2013) reported similar trend in LiP production by *Ganoderma lucidum* under SSF. Further increment in inoculum size above optimum inoculum size at 4 mL caused at least 50% of reduction in LiP activity. *Phanerochaete chrysosporium* showed maximum LiP production in the corncobs medium at 2 ml conidial suspension (Asgher et al., 2006). The above studies suggested that LiP activity is significantly affected by inoculum size. It may be explained that inoculum size lower than optimal level may not be sufficient to initiate the growth (Vantamuri and Kaliwal, 2016).

5.2.4 Effect of initial pH on LiP production

The initial medium pH for optimal LiP activity was investigated within pH 6-9 and the results are presented in Figure 5.5. The optimal pH for production of extracellular LiP by the tested strain was found to occur at pH 7; no appreciable LiP production was observed at pH 6.0. In fact, acidic pH did not favour LiP enzyme production. The production of extracellular LiP by *Bacillus licheniformis* strain was decreased to 90% and 56% of maximal activity at pH 8.0 and 9.0 respectively.

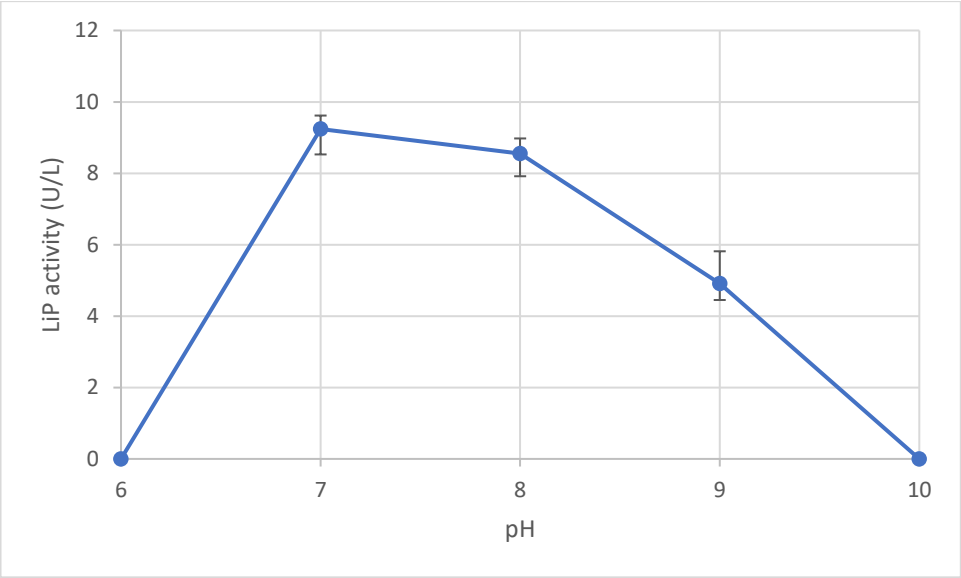

Figure 5.5 Effect of initial pH on LiP production by B. licheniformis for 15 h at 50°C, 16% (v/v) inoculum size under SmF cultivation using MSM-KL medium supplemented with 1% (w/v) glucose, 0.25 % (w/v) yeast extract, 0.05% (w/v) KL, 0.05% (w/v) $CaCl_2$.

From the analysis of the result, the capability of *B. licheniformis* produce LiP above pH 7 to pH 9 evident that LiP from this strain could be alkaliphilic. This finding concurs with the works of Falade et al. (2019a) and Rob et al. (1997) in which maximum peroxidase activity by *Ensifer adhaerens* and *Streptomyces avermitilis* UAH30 was recorded at pH 7.0 and 7.5 respectively. Moreover, it was found that alkali region favours peroxidase production by bacteria isolates (Shi et al., 2013b). Tuncer et al. (2004) and Falade et al. (2019d) reported optimum peroxidase activity from *Streptomyces* sp. F2621 and *Bacillus* sp FALADE-1 at pH 8.0. Similarly, *Bacillus* sp. SHC1 and *Luecobacter* sp. SHC3 recorded optimum production of ligninolytic enzymes at pH 8.0 (Abdul Rahman et al., 2013). In contrary, *Brevibacillus* and *Raoultella ornithinolytica* that showed optimum production at acidic pH 3.0 and 5.0. respectively. In fact, most fungal isolates also achieving optimum production at acidic condition (Rekik et al., 2019, Irshad and Asgher, 2011). Falade et al. (2019b) suggested that the optimal pH maybe genus specific since the peroxidase production varies across strains. Moreover, medium pH has significant effect on the electric charge on microbial cell which consequently influence the nutrients absorption and microbial growth (Briandet et al., 1999).

5.2.5 Effect of glucose concentration on LiP production

To investigate the optimal concentration of glucose on LiP activity from *B. licheniformis*, the tested bacteria was cultured on MSM-KL media (pH 7) with varying glucose concentration (0, 0.2, 0.4, 0.6 and 0.8% (w/v)). Figure 5.6 illustrates the effect of glucose concentration on LiP production by *B. licheniformis* under SmF at 50°C under static condition. Based on the result shown in Figure 5.6, about 27% of maximal LiP activity (3.42 U/L) was observed in medium without addition of glucose. Figure 5.6 shows LiP production is dependent on the presence of glucose in the growth medium. With the addition of glucose, the LiP production is continuous to increase and reached maximal LiP activity of 12.62 U/L at 0.4% (w/v) of glucose. However, further increase in glucose concentration above 0.4% (w/v) caused decline in LiP activity.

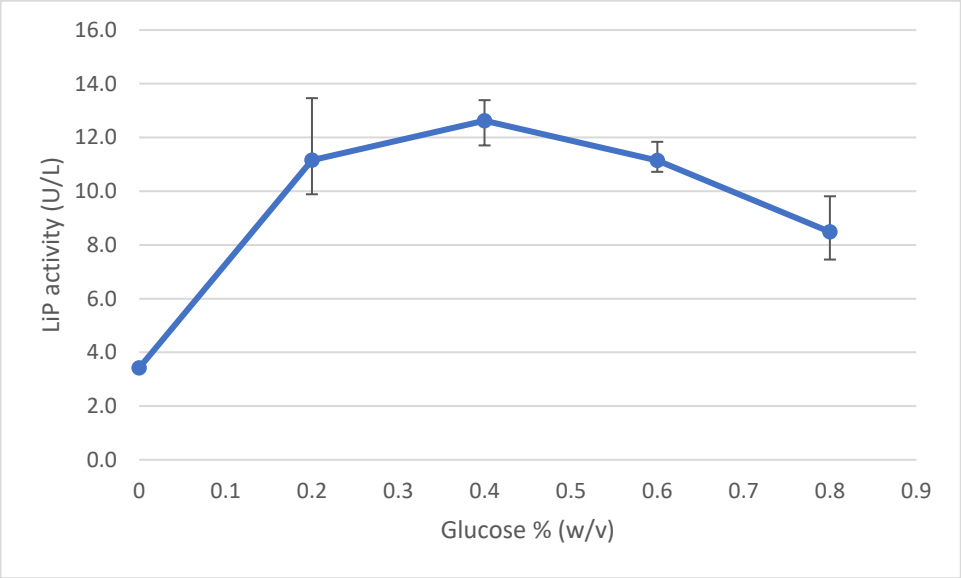

Figure 5.6 Effect of glucose concentration on LiP production by B. licheniformis for 15 h at 50°C, 16% (v/v) inoculum size under SmF cultivation using MSM-KL medium (pH 7) supplemented with 0.25 % (w/v) yeast extract, 0.05% (w/v) KL, 0.05% (w/v) $CaCl_2$

The production of LiP in the absence of glucose suggested that *B. licheniformis* could degrade and use highly recalcitrant lignin as the sole carbon source. Moreover, numerous newly isolated bacteria such as *Streptomyces* sp. S6, *Nonomuraea gerenzanensis* and *Pseudomonas* sp. strain Y6 have the potential in lignin degradability when growing in medium containing kraft lignin as sole carbon source (Riyadi et al., 2020, Casciello et al., 2017, Yang et al., 2017). Addition of 0.4% (w/v) of glucose also significantly stimulate the glucose by about 3 fold. Previous studies

have reported that several ligninolytic bacteria, including *Streptomyces griseosporeus* SN-9 and *Streptomyces viridisporus* T7A required glucose as additional carbon sources to stimulate LiP production. Similarly, glucose was reported as the best carbon source in ligninolytic enzymes production by *Trametes versicolor* IBL-04 (Iqbal et al., 2011) and *Schizophyllum commune* (Irshad and Asgher, 2011). According to Maria B. Pasti (Pasti et al., 1991), addition of glucose as secondary caron source changed the assimilation of nutrient in culture *Streptomyces*, D-glucose was used in preference to amino acid and significantly increase the LiP activity. Moreover, glucose concentration is critical in determining the maximal LiP activity. (Rekik et al., 2015) found that optimum glucose concentration in producing LiP from *Streptomyces griseosporeus* was reported at 1.5% (w/v). *Schizophyllum commune* IBL-06 and *Streptomyces viridosporeus* T7A also required carbon rich medium to stimulate LiP production. In contrast, Farrell et al. (1989) and Pasti et al. (1991) reported low glucose concentration at 1.0 and 0.5% (w/v) favoured LiP production by *Phanerochaete chrysosporium* BKM-F-1767 and *Streptomyces chromofuscus* A2 (Farrell et al., 1989, Pasti et al., 1991). The result revealed that low glucose concentration favoured LiP production by *Bacillus licheniformis*. Previous studies showed variation in optimum glucose concentration suggested the level of LiP produced is strain and medium dependent.

5.2.6 Effect of yeast extract concentration on LiP production

The effect of yeast concentration on LiP production was assessed by incubating *B. licheniformis* in MSM-KL medium under pre-optimal condition (15 h, 50°C, 16% (v/v) inoculum size; pH 7; 0.4% (w/v) glucose concentration) at varying yeast concentration at 0, 0.2, 0.4, 0.6 and 0.8% (w/v). Based on the result in Figure 5.7, The highest LiP activity was recorded in media supplemented with 0.2% (w/v) yeast extract (13.44 U/L), but higher concentrations led to decrease of LiP activity. In the present study, *Bacillus licheniformis* recorded 9.09 U/L, 5.88 U/L and 6.26 U/L of LiP activity at yeast extract concentration of 0.4% (w/v), 0.6% (w/v) and 0.8% (w/v) respectively. However, media without yeast extract supplement showed no LiP production. The above result demonstrated Yeast extract plays an important role in LiP induction by *Bacillus licheniformis*. This agrees with several studies by Falade's team where the presence of yeast extract enhanced the production of LiP enzyme (Falade et al., 2019d,

Falade et al., 2019a, Falade et al., 2019b). Moreover, yeast extract is the nitrogen source in MSM-KL medium. Nitrogen is a vital nutrient for microbial growth and synthesis of amino acids, enzyme cofactors, purines and pyrimidines. In addition, the production of ligninolytic enzymes is dependent on nature of the nitrogen source (Sivakani et al 2012). Jadhav et al. 2008 reported that the presence of organic nitrogen sources involved in NADH regeneration, which acts as electron donor in metabolic pathways of microbes and aids in peroxidase production.

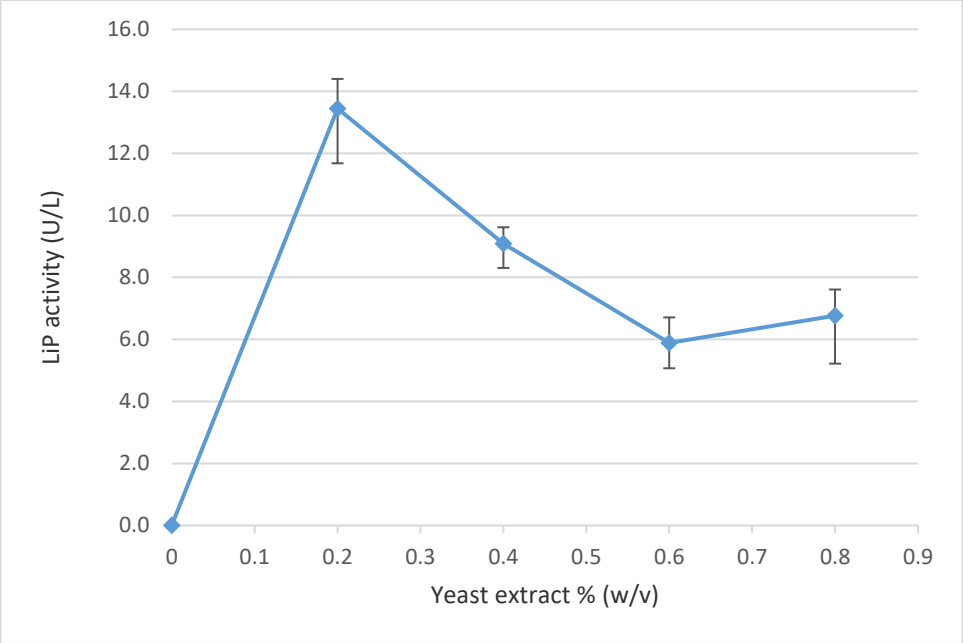

Figure 5.7 Effect of yeast extract concentration on LiP production by B. licheniformis for 15 h at 50°C, 16% (v/v) inoculum size, 0.4% (w/v) glucose concentration under SmF cultivation using MSM-KL medium (pH 7) supplemented with 0.05% (w/v) KL, 0.05% (w/v) $CaCl_2$

The optimal yeast extract concentration in the present study was similar to that reported for *Streptomyces griseosporeus* SN 9 (Rekik et al., 2015), *Streptomyces* K37 (Nour El-Dein et al., 2014) at about 0.3% (w/v) and 0.2% (w/v) respectively. However, Rob et al. (1997) reported optimal peroxidase production at 0.6 % (w/v) of yeast extract by *Streptomyces avermitilis* UAH30 (Rob et al., 1997). Yeast extract concentration above the optimal level may lead to inhibitory effect on peroxidase production due to high concentration of toxic compounds produce during the fermentation process (Nour El-Dein et al., 2014). It was reported that nitrogen limited condition favoured LiP production by LiP producer fungi, *Phanerochaete chrysosporium* (Farrell et al., 1989, Podgornik et al., 2001). The differences in the optimal level on LiP enzyme production across the previous studies suggested that it is strain dependent. Varying C:N ratio from its optimal level may induce different

mechanisms of LiP production and result in inhibition of growth and peroxidase production. As suggested by Nour El-Dein et al. (2014), the inhibition on peroxidase is probably due to high concentration of toxic compounds by-products during fermentation process but the type of toxic compounds was not determined. Further investigation is required to confirm this claim (Nour El-Dein et al., 2014).

5.2.7 Effect of lignin concentration on LiP production

The concentration of kraft lignin for optimal LiP production by *B. licheniformis* was investigated by altering the kraft lignin concentration from 0-0.1% (w/v) while incubating in MSM-KL medium (pH 7) at 50°C for 15 h under following fixed condition: SmF; 15 h; 50°C; 16% (v/v) inoculum size; 0.4% (w/v) glucose; 0.05% (w/v) KL; and 0.05% (w/v) $CaCl_2$. The result on LiP production at different concentration of lignin substrate was illustrated in Figure 5.8. Interestingly, the results revealed that LiP (4.81 U/L) was produced at 0% (w/v) of kraft lignin. Meanwhile, the LiP production increased continuously and recorded a peak LiP activity of 14.58 U/L in the presence of 0.8 g/L of kraft lignin. However, further increment of kraft lignin to 1.0 g/L cause reduction of about 25% of maximal yield in LiP activity.

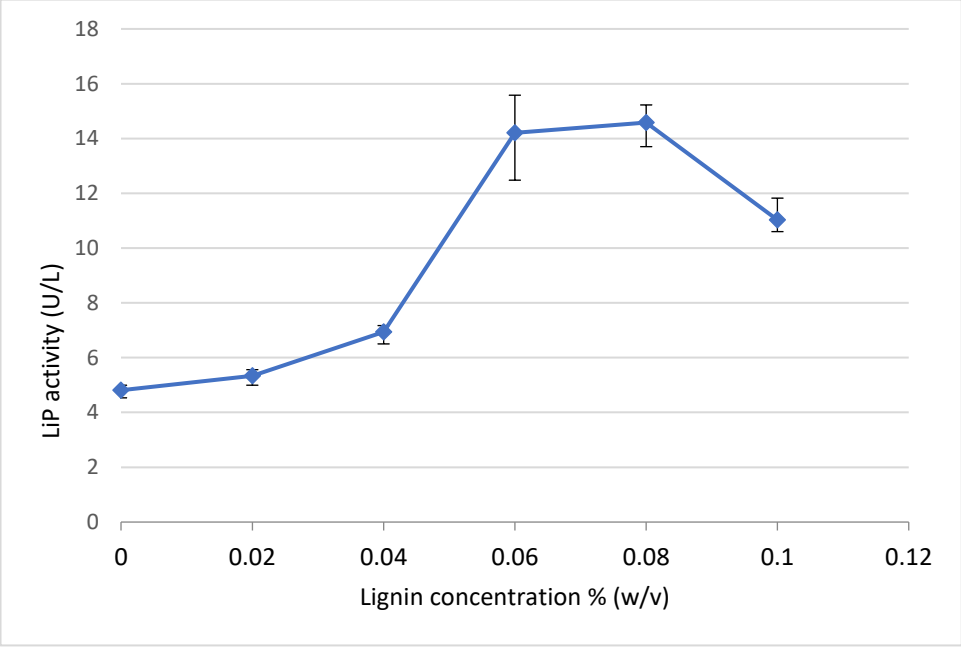

Figure 5.8 Effect of lignin concentration on LiP production by B. licheniformis for 15 h at 50°C, 16% (v/v) inoculum size, 0.4% (w/v) glucose, 0.2% (w/v) yeast extract under SmF cultivation using MSM-KL medium (pH 7) supplemented with 0.05% (w/v) $CaCl_2$

The result revealed that *B. licheniformis* is able to produce LiP in the absence of kraft lignin. This observation indicated *B. licheniformis* can utilise glucose as sole carbon source in LiP production. Similarly, previous studies on lignin degraders, *Phanerochaete chrysosporium* and *Phlebia radiata* were able to produce LiP enzyme in the absence of kraft lignin or any wood containing carbon source (Wang et al., 2008, Rogalski et al., 1991). Pasti et al. (1991) also demonstrated the induction of peroxidase by bacteria such as *Strepomyces chromofuscus* A2 and *S. viridosporus* T7A in the absence of lignin. In contrast, studies by Levin and Forchiassin (2001) and Casciello et al. (2017) showed that *Trametes trogii* and *Nonomuraea gerenzanensis* both require wood-containing medium or kraft lignin in producing LiP. The reduction in LiP activity at lignin concentration above 0.08% (w/v), could be due to the inhibition effect of some lignin phenolic or non-phenolic model by-products such as ferulic acid, p-coumaric acid, gallic acid, syringic acid, vanillin and vanillic acid (Abdul Rahman et al., 2013, Mathibe et al., 2020). Moreover, Eom and Kim (2014) also showed that 10 μM of free-phenolic compound could reduce the LiP activity by 10-30% (Pham et al., 2014).

Previous observations for optimal lignin concentration for LiP production were limited, but several studies showed that LiP production is affected by the presence of lignin. Abdul Rahman et al. (2013) reported that 0.8 g/L of oil palm EFB might inhibit the growth of lignin peroxidase producer such as *Leucobacter*, *Ochrombacte*r and *Bacillus,* but there was a lack of further study on effect of lignin concentration on LiP production. Pasti et al (1991) examined the effect of addition of lignocellulose on peroxidase production in medium F and medium F containing glucose as carbon source. Addition of lignin caused slight increment in peroxidase production by *Streptomyces chromofuscus* A2 and *S. viridosporus* T7A in F medium whereas negative effect was observed in glucose containing F medium (Pasti et al., 1991). However, there is dearth of information on optimal kraft lignin concentration on LiP production by bacteria through submerged fermentation.

5.2.8 Effect of $CaCl_2$ concentration on LiP production

Metals play an important role in enzyme production by affecting the microorganism growth and consequently affect the enzyme production (Nie and Aust, 1997, Chauhan, 2019). Some previous studies have shown that peroxidase production

is correlated with supplementation of calcium (Munir et al., 2015, Pazla et al., 2020, Uozumi et al., 1992). Pazla et al. (2020) and Macedo et al. (1999) reported supplementations of calcium show positive effect on lignin peroxidase production by *Phanerochaete chrysoporium* and *Streptomyces viridisporus* T7A. However, inhibitory effect of calcium on laccase was observed in *Pleurotus eryngii*. Wide difference in effect of calcium on enzyme production, thus, the concentration of $CaCl_2$ for optimal LiP production by *B. licheniformis* was investigated by altering the $CaCl_2$ concentration from 1.36- 6.80 mM while incubating in MSM-KL medium (pH 7) at 50°C for 15 h under following fixed condition: SmF; 15 h; 50°C; 16% (v/v) inoculum size; 0.4% (w/v) glucose; 0.08% (w/v) KL. The obtained results (Figure 5.9) revealed that calcium progressively induced activity of LiP to a certain concentration of about 4.08 mM where the peak of activity was detected (14.30 U/L). Further concentration increase caused about 16% (12.12 U/L) and 32% (9.72 U/L) reduction in LiP activity at 5.44 mM and 6.8 mM respectively (Figure 5.9).

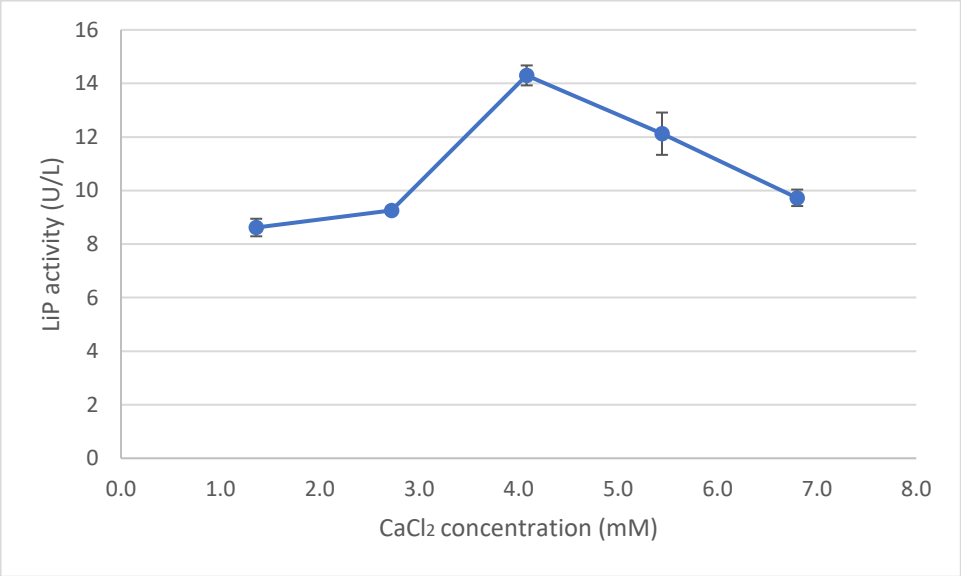

Figure 5.9 Effect of $CaCl_2$ concentration on LiP production by B. licheniformis for 15 h at 50°C, 16% (v/v) inoculum size, 0.4% (w/v) glucose, 0.2% (w/v) yeast extract, 0.08% lignin under SmF cultivation using MSM-KL medium (pH 7) at varying $CaCl_2$

Comparable optimum calcium concentration was also reported in peroxidase production *Trametes gibbosa* at 5 mM calcium concentration. Another study by Chauhan (2019) demonstrated maximal peroxidase production by *Grammothele fusca* at lower calcium concentration which is about 0.09 mM. Knežević et al. (2018) suggested stimulating effect of calcium cation on LiP activity could be attributed to the requirement of calcium in maintaining the integrity of peroxidase active site and

stabilise enzyme activity. It has been reported that peroxidase enzyme stability correlates with the release of the structural Ca^{2+} embedded in their structures (Semba et al., 2015), namely proximal and distal Ca^{2+}. One calcium ion was reported to be tightly bound on proximal side of heme, while the other one loosely bound on the distal side of heme (Gao et al., 2016). Removal of the Ca^{2+}, from MnP and LiP from *P. chrysosporium* resulted in total loss of activity. Study by Nie and Aust (1997) reported loss of Ca^{2+} in thermally inactivated LiP and 92% of LiP activity was recovered upon addition of 2 mM Ca^{2+} with the increment of 1.2 mol of Ca^{2+}/ mol of LiP to 4.4 mol of Ca^{2+}/ mol of LiP (Nie and Aust, 1997). Similar observation was also demonstrated by Gao et al. (2016) whereby Versatile peroxidase that contained greater Ca^{2+}/ mol protein exhibited greater enzyme activity and thermal stability.

Apart from that, supplementation of calcium may stimulate cell growth or affect the cell morphology that favoured the uptake of nutrients thus leading to greater enzyme production (Knežević et al., 2018, Febrina et al., 2016). In fermentation environment, all aerobic organisms must cope with oxidative stress, which may induce cell damage. It was suggested that Ca^{2+} has the regulatory effect on putative Ca^{2+} binding protein that is related with cell adaptive response or cell guarded status of the bacterial cell which subsequently affect the bacteria growth. Huang and Chen (2013) demonstrated 5 mM of Ca^{2+} increase heat resistance of Lactic acid Bacteria (LAB) by showing greater cell survival and shortest cell regrowth time (Huang and Chen, 2013). Similarly, Herbaud et al. (1998) also reported immediate growth of *Bacillus subtilis* and subsequently affect the β-galactosidase enzyme activity after addition of Ca^{2+} into Luria Broth medium containing Ethylene glycol-bis(β-aminoether)-N,N,N',N'-tetraacetic acid (EGTA) that suppressed the bacteria growth (Herbaud et al., 1998).

Overall, the current study showed that addition of calcium ion in fermentation medium does increase the enzyme activity of LiP. Thus, it is believed that calcium ion may have regulatory effect on growth of *Bacillus licheniformis* and LiP activity. Hence, it is interesting to determine role of Ca^{2+} ion in regulating LiP activity and the growth of *Bacillus licheniformis* since this information would provide a guide for engineering LiP towards higher thermal stability and enzyme production.

In conclusion, LiP production by *Bacillus licheniformis* CLMTCHB29 was optimal at 50℃ , neutral pH, 16% (v/v) inoculum size culture and relatively short incubation time

at 15 h. The nutritional condition changes include 0.4% (w/v) glucose, 0.2% (w/v) yeast extract, 0.08% (w/v) kraft lignin and 0.06% (w/v) $CaCl_2$. Both glucose and kraft lignin were required to stimulate the LiP production. Besides, the presence of yeast extract was also critical in LiP production. The optimum conditions resulted about 40% of increment in LiP production from 10 U/L to 14 U/L. Based on the ANOVA analysis shown in Appendix C, the result obtained in LiP production via OFAT are considered statistically significant since *P* values for each parameters are less than 0.05.

5.3 FTIR analysis on degradability of *Bacillus licheniformis* on kraft lignin

In the degradation study, The culture of *Bacillus licheniformis* CLMTCHB29 was grown under optimal nutritional and culture condition obtained from section 5.2 (incubation at 50°C under SmF cultivation using MSM-KL medium (pH 7) supplemented with 16% (v/v) inoculum size, 0.4% (w/v) glucose, 0.2% (w/v) yeast extract, 0.08% (w/v) lignin and 4.08 mM $CaCl_2$) for 96 h. Another Erlenmeyer flask of MSM-KL medium without *Bacillus licheniformis* CLMTCHB29 was incubated under similar condition and served as control which denoted as untreated lignin. The kraft lignin degradation ability by *Bacillus licheniformis* CLMTCHB29 was determined by examine the difference between the structure of treated and untreated kraft lignin in terms of chemical bonding related to lignin structure such as guaiacyl, syringyl and lignin subunits through FTIR analysis. Figure 5.10 shows the FTIR spectra of treated lignin by *B. licheniformis* for 96 hr and untreated lignin served as control. In Figure 5.10, a strong absorption was observed around 3400 cm^{-1}, which is assigned to the stretching vibration of aromatic and aliphatic-OH in the lignin (Mousavioun et al., 2012) whereas 2990 cm^{-1} which is assigned to the stretching of the vibration of C-H band in CH_2, CH_3 and CH_3O groups of the lignin structure (Sun et al., 2013). Slight reduction was observed on the absorption peaks of the bands at 3400 and 2990cm^{-1} in the treated sample compared with the untreated sample, indicating that some methoxyls maybe removed from the aromatic ring during the biodegradation process. This phenomenon agrees with the study by on biodegradation process of lignin by *Penicillium simplicissimum* (Liu et al., 2014). In addition, there are also existed many well-defined peaks in the fingerprint region between 1700 cm^{-1} and 900

cm^{-1} which was illustrated in Figure 5.11 and 5.12. In this fingerprint region, more clear differences can be detected in the infrared spectra, both in the absorption and shape of the bands.

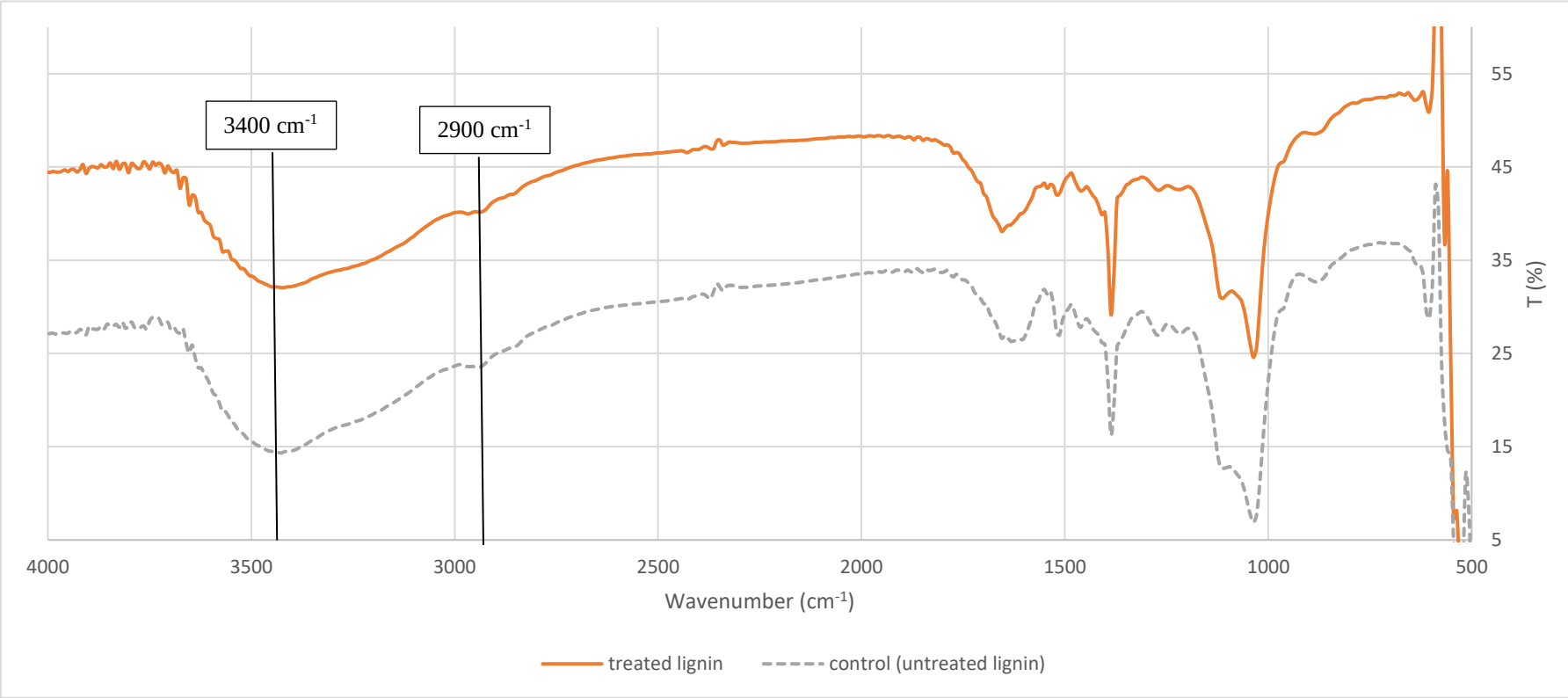

Figure 5.10 FTIR spectra of treated lignin with B. licheniformis for 96 h and control sample. Slight reduction was observed on the absorption peaks of the bands at 3400 and 2990cm^{-1}. These peaks are related to the stretching vibration of aromatic and aliphatic-OH in the lignin and C-H band in CH_2, CH_3 and CH_3O groups of the lignin structure respectively.

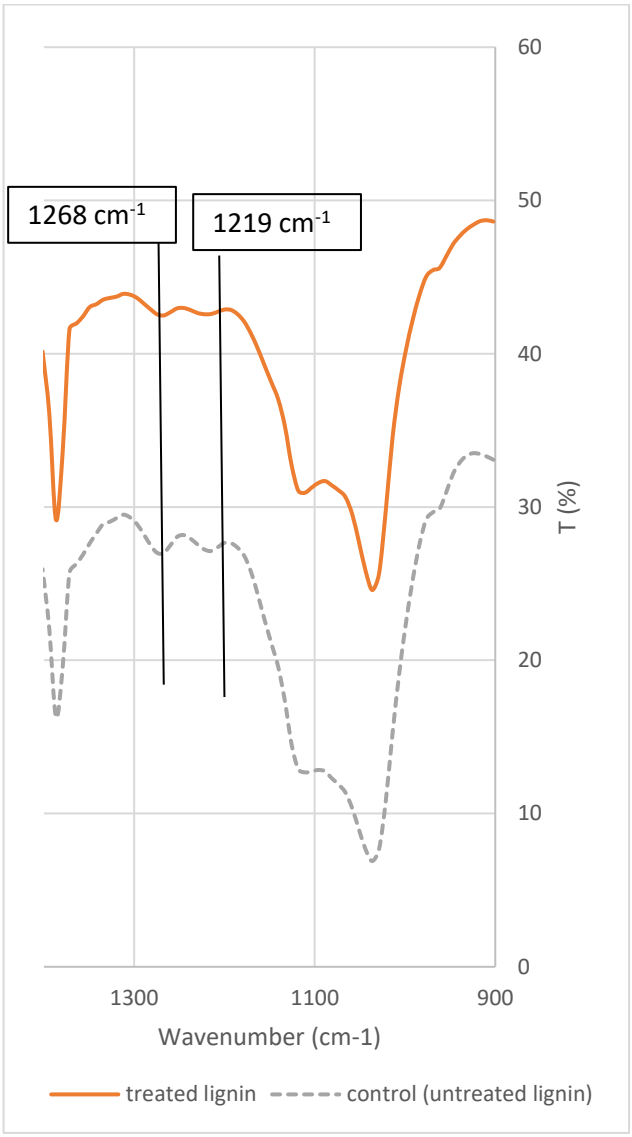

Figure 5.11 FTIR spectra of treated lignin with B. licheniformis for 96 h and untreated lignin served as control sample at region from 1400 to 900 cm^{-1}. Decrement was observed in the peak values at 1219 cm^{-1} and 1268 cm^{-1}. These peaks are attributed to C-C, C-O and C=O stretching; C-O linkage in guaiacyl aromatic methoxyl group and C-O stretching in lignin respectively.

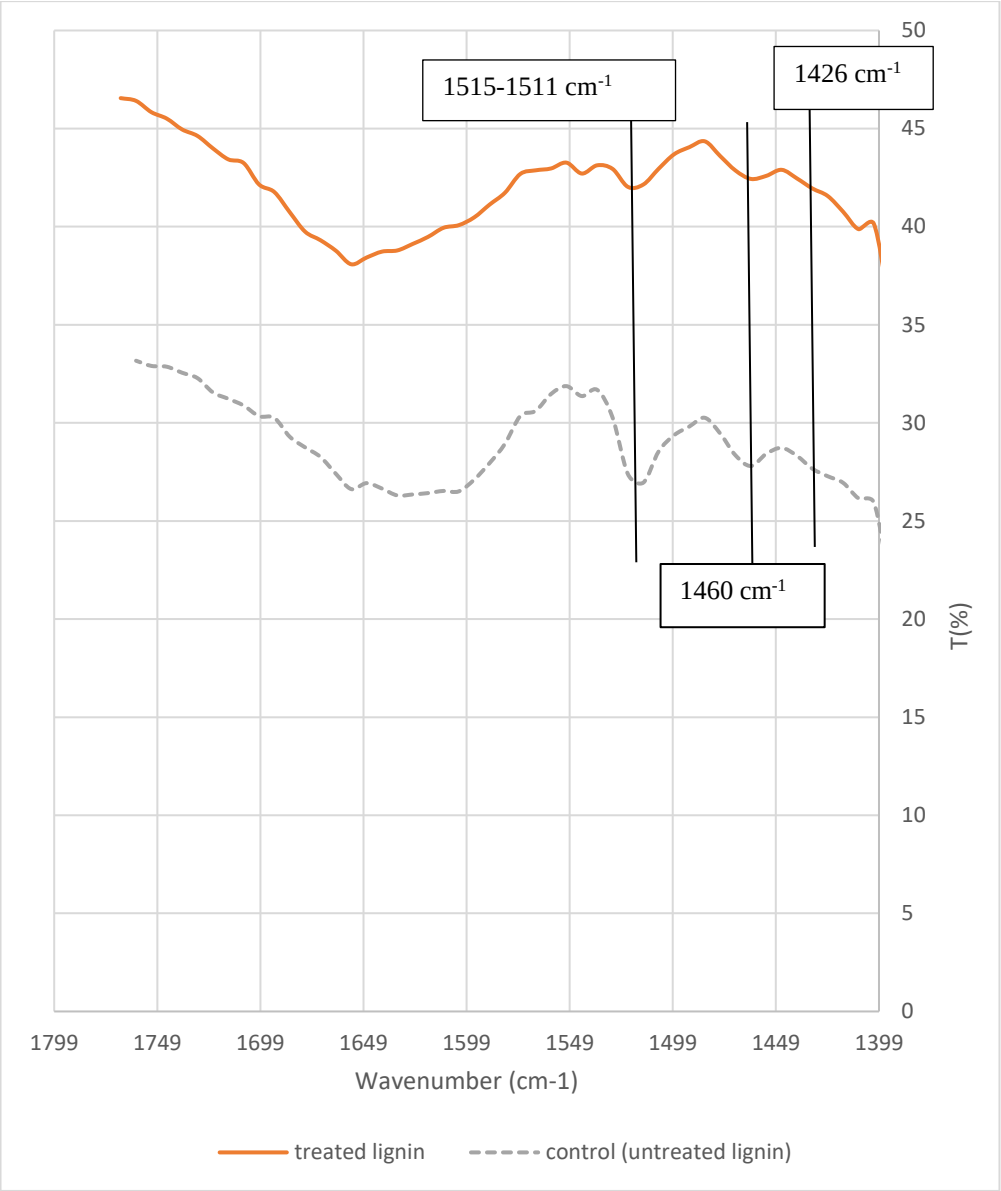

Figure 5.12 FTIR spectra of treated lignin with B. licheniformis for 96 h and untreated lignin served as control sample at region from 1800 to 1400 cm^{-1}. Decrement in transmittance value was observed in the peak related to functional group of lignin subunit (1515-1511 cm^{-1}, 1460 cm^{-1} and 1426 cm^{-1})

The functional groups of the observed peaks were summarised in Table 5.1. Based on the transmittance result (Table 5.1), decrement was observed in the peak related to functional group of lignin subunit (1515-1511 cm^{-1}, 1460 cm^{-1} and 1426 cm^{-1}). In fact, these are the major peaks representing the main functional group in lignin structure. The obvious difference can be found in Fig. 5.12 where the sharp peak in the range of 1515-1511 cm^{-1}, 1460 cm^{-1} are remarkably reduced and tiny peak at 1426 cm^{-1} is barely visible in treated sample as compared to control sample. The peak values at 1219 cm^{-1} and 1268 cm^{-1} are attributed to C-C, C-O and C=O stretching; C-O linkage

in guaiacyl aromatic methoxyl group and C-O stretching in lignin respectively. Slight decrement in the band's height was observed at these peaks and this may indicate reduction of C-O stretch or C-O linkage in lignin or guaiacyl unit.

Gelbrich et al. (2012) reported the absorbance value of wavenumbers at 1515-1511 cm^{-1} and 1268 cm^{-1} in the lignin are correlated linearly with the lignin contents. Particularly, the absorbance at wave number 1515- 1511 cm^{-1} was found to have high correlation with the C=C stretching of the aromatic ring in lignin since this absorbance would not be influenced by another functional groups through overlapping effect (Pandey, 1999). Thus, the degradation of the lignin content was calculated based on the transmittance value at 1515-1511 cm^{-1} as suggested by Liu et al. (2014). The degradation percent rate was governed by the following equation.

$$\%\ of\ degradation = \frac{T_1 - T_0}{T_0}\ x\ 100\%$$

T_1 = transmittance value at 1515-1511 cm^{-1} in treated lignin sample
T_0 = transmittance value at 1515-1511 cm^{-1} in non-treated lignin sample

The degradation percent rate was 56.3% at peak value 1515-1511 cm^{-1}. Generally, significant reduction was observed at chemical bonding related to guaiacyl, syringyl and lignin subunit. The changes in these functional groups may be due to the decomposition of lignin structure by *Bacillus licheniformis*. Thus, this inferred that ligninolytic enzymes from newly isolated thermophiles *Bacillus licheniformis* strain CLMTCHB 29 has the potential in degrading lignin content in kraft lignin powder after 96 hr of incubation in MSM-KL medium. However, further analysis of lignin substrates and aromatics compound presence in the liquid fermentation medium using Liquid chromatography would be required to confirm the possibility of decomposition of kraft lignin (Levy-Booth et al., 2021).

Table 5.1 functional group in untreated lignin (control) and treated lignin at selected wavenumber

Wave number (cm^{-1})	Functional group	Transmittance (%)	
		Untreated lignin (control)	Treated lignin
1123-1110	Aromatic C-H deformation of syringyl units	12.68	30.93
1219	C-C, C-O and C=O stretching of guaiacyl unit	27.23	45.57
1268	C-O stretching (lignin) and for C-O linkage in guaiacyl aromatic methoxyl groups	27.00	42.50
1426	C-H deformation (lignin) (phenol-ether bonds of lignin)	27.28	41.56
1460	C-H bending of methyl and methylene groups (asymmetric bendings in CH3 in lignin)	27.80	42.43
1515-1511	C=C stretching of aromatic ring (lignin) (aromatic skeletal vibration in lignin)	26.95	42.15
1615-1600	C=C stretching of aromatic ring (lignin) (aromatic skeletal vibration in lignin)	26.51	40.08

Chapter 6 Purification and Characterisation of Lignin Peroxidase

In chapter 6, it presents about the quantitative result on the purification of LiP from the culture supernatant. Apart from that, the purified LiP was characterised with a series of characterisation to determine its basic properties such as kinetic study, pI isoelectric point, substrate specificity, temperature and pH stability and effect of metal ions and inhibitors on LiP. This characteristic revealed the suitability and stability of LiP for industrial application.

6.1 Enzyme isolation and purification

In the enzyme purification, ultrafiltration on a 10kDa-cut off membrane and Biogel P-100 chromatography were used to purify LiP from the supernatant of crude enzyme.

6.1.1 Ultrafiltration

In the ultrafiltration step, 500 mL of culture supernatant containing 18.92 U of LiP with 4.47 mg of protein (spec. activity 4.23 U/mg) was concentrated approximately 62.5-fold to 8 mL of ultrafiltrate retentate contained 1.62 U of LiP with 0.52 mg of protein (spec. activity 3.10 U/mg) under room temperature. The recovery yield of LiP in retentate is about 8.6% with 0.73-fold of purification yield (Table 6.1). The LiP activity of UF filtrate was determined as 12.26 U in 492 mL of filtrate. About 8.6% of LiP enzyme activity was recovered in the ultrafiltration retentate and 64.8% of LiP enzyme activity remained in the ultrafiltration filtrate with 26.6% enzyme activity loss. The low recovery in ultrafiltration steps show major loss of LiP in the filtrate. This is likely due to low retention efficiency of Ultracel Regenerated Cellulose membrane (cut-off weight at 10 kDa) in retaining LiP with 26 kDa molecular weight. This could be overcome by selecting an ultrafiltration membrane with a lower cut-off weight such as 5 kDa. The cause of loss of enzyme activity is unknown. The lower activity of the purified enzyme observed could be due to the loss of enzyme cofactor

during the purification. Several studies reported metalloproteins structures are associated with metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} (Waldron et al., 2009, Patel et al., 2021). Mn^{2+} was reported as a powerful activators in increasing functional stability of HRP and stabilize the enzyme structure along with its long term stability (Mahmoudi et al., 2003). Besides, Ca^{2+} is also an important metal ions in preventing thermal inactivation of lignin peroxidase from *Phanerochaete chrysosporium* (Nie and Aust, 1997). These study demonstrated metal ions play an important cofactors in maintaining the functionality of peroxidase . However, the role of metal ions as an enzyme cofactor in maintaining the LiP activity in the current study can only be verified through the elucidation of the chemical and molecular structure of the purified LiP. Thus, further investigation on the metal ions concentration and the conformational change of LiP structure in retentate and filtrate could be examine to determine the possible reason on the enzyme activity loss.

The operating conditions of ultrafiltration unit in this study was operated at recommended conditions according to the standard protocol provided by the manufacturer. Study by Greening and Simpson (2010) demonstrated differing membrane type and membrane orientation of the filter unit has direct impact on the transmembrane pressure and permeability of each membrane which in turn affects the selectivity of protein separation (Greening and Simpson, 2010). Apart from that, Kim et al. (1993) also reported that the membrane properties coupled with the operating conditions such as operating pressure, stirring speeds, pH solution, protein concentration contributed to the protein transmission through ultrafiltration membrane (Kim et al., 1993). Thus, as suggested by Greening and Simpson (2010), optimisation on the above factors on ultrafiltration efficiency can be considered to enhance the protein recovery in ultrafiltration process.

Table 6.1 LiP activity and its recovery in crude enzyme, retentate and permeate of ultrafiltration

	LiP activity (U/L)	Recovery (%)
Retentate	1.62	8.6
Filtrate	12.26	64.8
Total	13.88	73.4

6.1.2 Biogel P-100 gel filtration chromatography

Gel filtration chromatography is a method where the proteins are separated according to their size. The gel filtration matrix contains pores which allow small proteins to enter while excluding larger ones. So, the first elute are the larger one followed by the smaller protein. Generally, molecular weight of a typical LiP was in the range of 36 kDa to 55 kDa, thus, the filtration gel matrix, Biogel P100 with a fractionation range of 5- 100 kDa was selected (Zeng et al., 2013, Vandana et al., 2019, Wadhwani et al., 2018).

Retentate solution obtained from ultrafiltration was subjected to Biogel P100 gel filtration chromatography (20 cm x 1 cm) based on molecular weight separation. 1 mL of ultrafiltration retentate equivalent to 0.3874 U/mg specific activity of LiP was loaded to Biogel P100 gel filtration and eluted at the speed of 4 mL/hr. The elution was collected at fraction of 1 mL. LiP is a typical heme-containing enzyme; thus, it may exhibit absorbance at 409 nm. The protein content and heme-protein content were measured at UV wavelength 280 nm and 409 nm respectively. In addition, LiP activity was assayed according to standard LiP assay method stated in section 2.3.1.1.

According to Figure 6.1, the protein elution pattern on the Biogel P100 gel filtration column shows seven protein peaks at 280 nm designated Peak 1 to Peak 7 Results for LiP activity revealed that activity was only associated with peak 4. The results of the purification steps are summarised in Table 6.2. The effectiveness of Biogel P100 chromatography in purifying LiP was estimated at 0.60- fold with a purification yield of 2.08% and a specific activity of 2.54 U/mg with veratryl alcohol. On the contrary, previous studies by Dawkar et al (2009) and Wadhwani et al. (2018) reported greater LiP purification factor of 42-fold and 959-fold respectively (Wadhwani et al., 2018, Dawkar et al., 2009). The low purification fold may be due to the loss of enzyme activity during the gel filtration chromatography process. Based on the elution profile of LiP from Biogel P100 chromatography column (Figure 6.1), the highly positive LiP fractions (fraction 13-15) were collected and pooled together for subsequent characterisation study. Although fractions 12 and 16 exhibited some residual LiP activity, they were not combined with the more active fractions to avoid further dilution of the purified LiP enzyme.

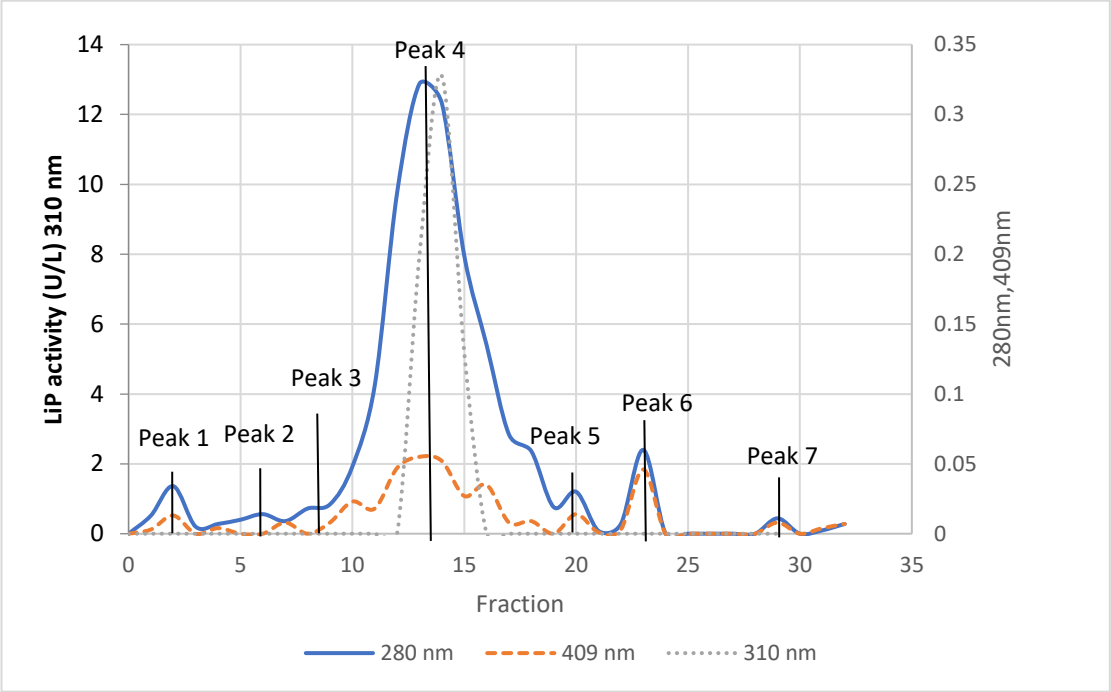

Figure 6.1 Elution profile of Biogel chromatography of partially purified LiP from Bacillus licheniformis and assayed for protein content at 280 nm; heme-protein content at 409 nm and LiP activity at 310 nm. Fraction 13-15 containing purified LiP were pooled together.

Table 6.2 Biogel P-100 gel chromatography purification profile of LiP from Bacillus licheniformis CLMTCHB 29

	Total activity (U)	Total protein (mg)	Volume (mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	18.9176	4.4737	500	4.2286	100.00	1.00
UF retentate	1.6163	0.5215	8	3.0994	8.54	0.73
Biogel P-100	0.3936	0.1547	24	2.5439	2.08	0.60

6.2 Molecular weight identification

Vacuum filtered crude enzyme sample (0.27 µg protein), ultrafiltration retentate (1.96 µg protein) and partially purified LiP (0.19 µg protein) was loaded onto SDS-PAGE to estimate the molecular weight of LiP. As shown in Fig. 5.2, no visible band was observed in crude enzyme (Lane 2), but 8 visible bands were observed in

ultrafiltration retentate fraction (Lane 3). Invisible band in vacuum filtred crude enzyme sample may be due to protein concentration per band is lower than the detection level (30 ng) of CBB G-250 staining method on SDS-PAGE. Partially purified fraction showed single band in SDS-PAGE on Lane 4 at 26 kDa indicating the reasonably high purity of LiP in fraction obtained from Biogel chromatography (Fig. 5.2). The finding in this study is similar with those reports on catalase peroxidase, RaoPrx and HaP2 from *Streptomyces* sp. strain AM2 that attained molecular weight of 11.45 kDa, 17.587 kDa and 25.17 kDa respectively (Falade et al., 2019d, Falade et al., 2019c, Fodil et al., 2011). However, the molecular weight of *B. licheniformis* LiP differed significantly from that of typical LiP purified from the fungus *Phanerochaete chrysosporium* (55 kDa), *Humicola grisea* (70 kDa) and *Bjerkandera adusta* strain CX-9 (45 kDa) (Vandana et al., 2019, Moubasher et al., 2017, Bouacem et al., 2018). Similarly, previous study on bacterial LiP isolated from *Acinetobacter* sp. SW30, *Pseudomonas* sp. and *Streptomyces griseosporeus* SN9 also showed relatively higher molecular weight ranged between 43 – 97.4 kDa compared to current study (Wadhwani et al., 2018, Kalyani et al., 2011, Rekik et al., 2015). Nonetheless, variation in the molecular weight is also observed in the same species whereby Zeng et al. (2013) and Vandana et al. (2019) reported 36 and 55 kDa of LiP respectively from fungi *Phanerochaete chrysosporium*.

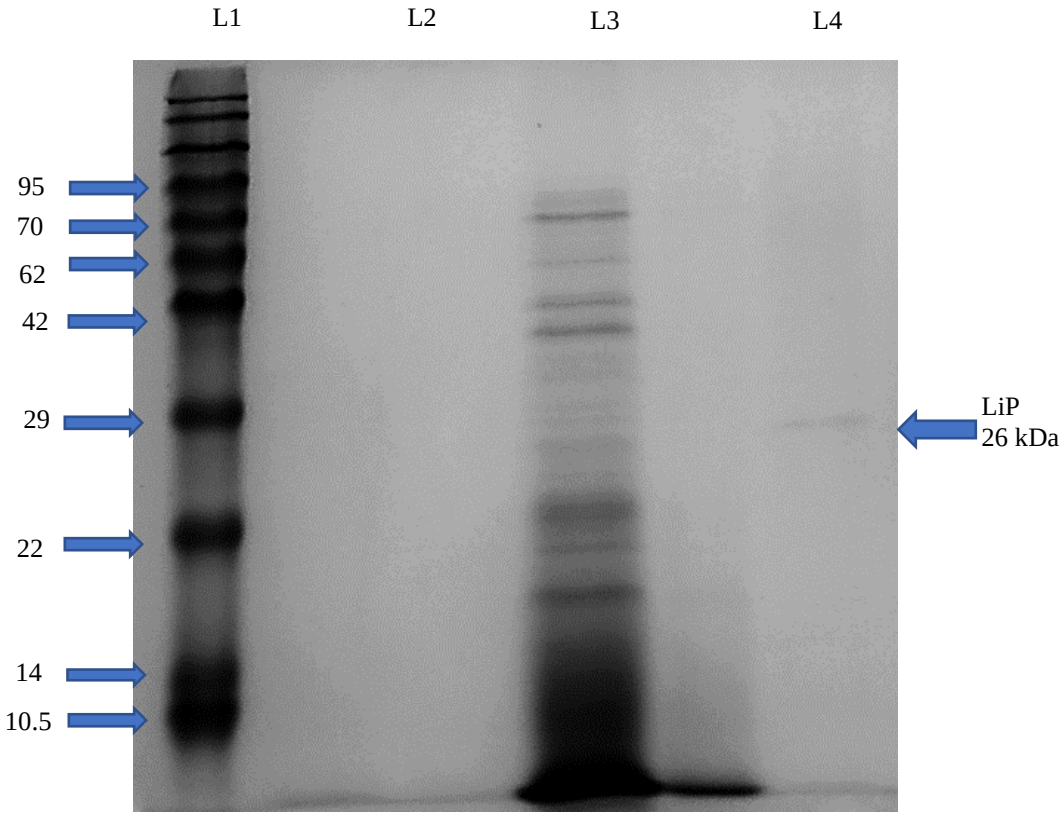

Figure 6.2 SDS of partially purified LiP Lane 1: Molecular weight marker (Chromatein Prestained Protein Ladder, Vivantis); Lane 2: Vacuum filtered crude enzyme; Lane 3: Retentate from ultrafiltration (Amicon); Lane 4: Fraction from gel filtration chromatography)

6.3 pI identification

The isoelectric point is commonly referred to as the pI whereby it is the pH value at which the protein exhibits net overall surface charge (Audain et al., 2015). A protein that is in a pH region greater than pI is negatively charge and lower than pI is positively charged. When the protein is at pH that is near or at pI value, the solubility of protein is typically minimal. Moreover, it also affects the stability of an enzyme. The enzyme would denature when the pH deviates from the physiological pH. Thus, information on pI of a protein is useful in selecting buffer solution used in the purification process and storage purposes. Apart from that, it is also important in understanding enzyme-substrate interactions (Xia, 2007). Figure 6.3 illustrates the zeta potential of partially purified LiP from pH 1.2 to 11.7 and it was observed that LiP

exhibited 0 mV at pH 1.56, thus the isoelectric point (pI) of the partially purified LiP was found to be 1.56. This result revealed LiP has minimal solubility at 1.56 and further implied LiP might exhibit lower enzyme activity at this pH. Study showed that this is probably due to the negatively charged substrate and the net charge on the enzyme might influence the steady state enzyme kinetics. This finding was significantly different from previous study on *RaoPrx*, *Sacharomonospora viridis* DSM 43017 DyP and *Sporotrichum* thermophile-like catalase-peroxidase gene recorded pI with 4.51, 5.17 and 7.01 respectively (Falade et al., 2019c, Yu et al., 2014, Falade et al., 2019d). To my best knowledge, this is the first report on LiP with extremely acidic pI value. Tokmakov et al. (2021) claimed that pI value is primarily defined by the amino acid composition of a protein sequence based on the combination of dissociation constant (pKa) values of the constituent amino acids. Thus, the low pI of LiP from classical LiP could be due to the difference in their amino acid composition in the enzyme structure (Tokmakov et al., 2021). However, further investigation on the enzyme structure is required to confirm this.

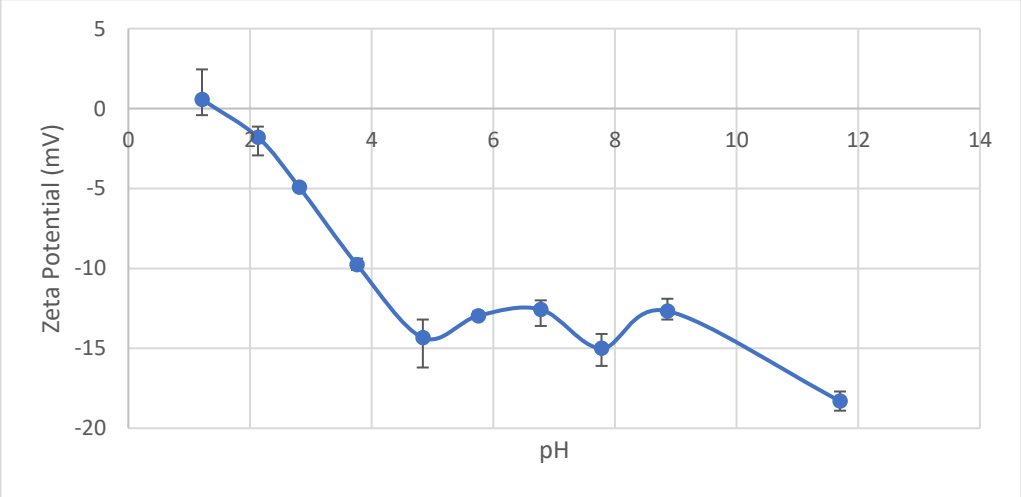

Figure 6.3 Zeta potential of LiP enzyme. Each point represents the mean result of three replicate with error bar indicating the maximum deviation from the mean value.

6.4 Kinetic constants of LiP (K_m and V_{max})

Kinetic study is an important characteristic indicating reaction rate of an enzyme subjected to the tested substrate. This information is important in determining

the usability of an enzyme as it describes the affinity of an enzyme towards substrate. The Michaelis constants, namely K_m and V_{max} for purified LiP were determined by measuring the corresponding enzyme velocity at varying concentration of veratryl alcohol and hydrogen peroxide. Based on the Michaelis Menten and Lineweaver Burk plots (Appendix B), the kinetic constants were evaluated. The purified LiP agrees with Michaelis Menten behaviour, with a K_m of 80 mM and V_{max} of 15.06 U mg^{-1} for H_2O_2 and a K_m of 0.7585 mM and V_{max} of 3.78 U mg^{-1} for VA (Table 6.3). The K_m value for purified LiP found in this study is relatively higher than those previous reports on fungus (*Phanerochaete chrysosporium, Irpex lacteus* and *Loweporus lividus* MTCC-1178) that showed K_m value ranging from 0.0136 mM – 0.333 mM (Ollikka et al., 1993, Rothschild et al., 2002, Yadav et al., 2009). The V_{max} for the purified LiP is lower than the reported V_{max} of LiP obtained from *Bjerkandera* sp. strain BOS55 and *Phanerochaete chrysosporium*. In contrast, the V_{max} for veratryl alcohol oxidation by LiP is significantly higher than PpDyP enzyme from *Pseudomonas putida* that reported V_{max} of 6.2 x 10^{-6} (Santos et al., 2014), while no kinetic activity was detected in BsDyP from *Bacillus subtilis.* Overall, the kinetic study result revealed that the purified LiP from *Bacillus licheniformis* is able to oxidise the classical LiP substrate (VA) although with lower binding affinity and reaction rate compared to fungal LiP, but possess greater binding affinity and reaction than bacteria DyP. In fact, there are very limited data on kinetic study of bacteria LiP enzyme on VA substrate since bacteria peroxidases (LiP, DyP) were assayed using phenolic compounds. For instance, RaoPrx was assayed with pyrogallol (Falade et al., 2019c). and Peroxidase from *N. gerezanesis* was assayed with ABTS, 2,6-DMP and catechol (Casciello et al., 2017). Nonetheless, kinetic features support that LiP is capable of degrading VA under room condition and provides some basic understanding on its substrate specificity toward VA.

Table 6.3 Michaelis constant Km and Vmax value of LiP from B. licheniformis CLMTCHB29 (VA and H_2O_2 substrate) on previous study and current study

Microorganisms	K_mVA (mM)	V_{max} (U mg^{-1})	$K_mH_2O_2$ (mM)	V_{max} (U mg^{-1})	Reference
Phanerochaete chrysosporium	0.123 - 0.333	-	0.127-0.235	-	(Ollikka et al., 1993)
Phanerochaete chrysosporium	0.083 – 0.200	-	0.085-0.140	-	(Glumoff et al., 1990)
Phanerochaete chrysosporium	0.100	15.2	-	-	(Zeng et al., 2013)
Irpex lacteus	0.2338 – 0.3264	-	0.0136-0.0166	-	(Rothschild et al., 2002)
Loweporus lividus **MTCC-1178**	0.058	-	0.083	-	(Yadav et al., 2009)
Bjerkandera **sp. strain BOS55**	0.059-0.089	13.9 – 15.9	0.442	-	(Ten Have et al., 1998)
Pseudomonas putida	-	6.2×10^{-6}	-	-	(Santos et al., 2014)
Bjerkandera **sp.**	1.5	13	0.182	28	(Moreira et al., 2006)
Bacillus licheniformis **CLMTCHB29**	0.7585	3.78	80	15.06	Current study

6.5 Substrate specificity

The purified LiP seems to have low substrate specificity since it has the ability to oxidise a variety of phenolic, non-phenolic compounds and dyes. Table 6.4 summarises the specific activity of LiP on substrate VA, ABTS, 2,6- DMP, Guaiacol, Malachite Green (MG), Azure B and Phenol Red. The relative substrate specificity activity for veratryl alcohol is in the following order: 2,6- DMP > ABTS > Guaiacol > VA > Malachite Green (MG) > Azure B > Phenol Red. However, no activity was observed with Congo Red. All tested phenolic compounds demonstrated greater specific activity towards LiP enzyme when compared to classic substrate, veratryl alcohol. The relative activity of phenolic compounds 2,6-DMP, ABTS and guaiacol are about 12-fold, 8-fold and 2-fold higher than VA respectively. Similar findings were

also observed in LiP from *Acinetobacter* sp. SW 30 and a heme peroxidase, LsaPOX from fungi *Leptogium saturninum*. This observation seems to suggest that LiP enzyme is able to degrade phenolic compound more readily than non-phenolic compound (Pollegioni et al., 2015, Chandra et al., 2017). However, VA appeared to be a preferred substrate in most of the fungal LiP although it shows reactivity toward phenolic compounds such as guaiacol and 2,6-DMP (Rothschild et al., 2002, Glumoff et al., 1990). The oxidation of 2,6-DMP and guaiacol by LiP is at least an order of magnitude higher than DyP and LiP from *Thermobifida fusca*, *Saccharomonospora viridis* DSM 43017 and *Acinetobater* sp. SW 30 respectively.

Table 6.4 Substrate specific activity of partially purified LiP on previous studies and current study

Microorganisms	Enzymes	Substrate	Specific activity (U mg^{-1})	Reference
Bacillus subtilis	DyP	ABTS	15	(Santos et al., 2014)
Pseudomonas putida MET 94	DyP	ABTS	40	
		VA	6.2 x 10^{-6}	
Bacillus subtilis	DyP	ABTS	66.80	(Min et al., 2015)
		VA	0.13	
Thermobifida fusca	DyP	VA	0.01	(van Bloois et al., 2010)
		Guaiacol	0.03	
		2,6-DMP	0.17	
Acinetobacter calcoaceticus	LiP	VA	0.043	(Ghodake et al., 2009)
Acinetobacter sp. SW 30	LiP	VA	0.337	(Wadhwani et al., 2018)
		Guaiacol	0.607	
Saccharomonospora viridis DSM 43017	DyP	VA	0.03	(Yu et al., 2014)
		2,6-DMP	0.06	
		Azure B	1.62 (pH 6)	
		Malachite Green	8.4	
Leptogium saturnium	Peroxidase	Azure B	0.03 (pH 3)	(Liers et al., 2011)
		2,6-DMP	53	
		VA	0.1	
Bacillus licheniformis	LiP	VA	3.75	Current study
		ABTS	31.64	
		2,6-DMP	46.29	
		Guaiacol	8.92	
		Azure B	1.87	
		Malachite Green	1.97 (pH 7)	
		Phenol Red	0.08 (pH 7)	

In general, LiP from *Bacillus licheniformis* has showed significantly higher VA oxidation as compared to LiP from *Acinetobacter* sp. SW 30 and *Acinetobacter calcoaceticus* for about 11-fold and 87-fold respectively. Besides, several DyP also demonstrated lower specific activity for substrate VA. In addition, not all DyP possess the ability to degrade VA. In fact, the LiP has high similarity with DyP concerning the affinity to high redox potential substrate such as VA and Azure B. Due to the complexity of Azure B structure, it can only be oxidised by high redox potential agents, particularly LiPs or DyPs (Aguiar and Ferraz, 2007, Haq and Raj, 2018). However, both enzymes have a distinct difference in the working pH whereby LiP is active under highly acidic condition as low as pH 2 but DyP functions at slightly acidic or close to neutral pH. Thus, it is further confirmed that the isolated peroxidase is a typical LiP since it is able to oxidise VA and Azure B at pH as low as pH 2.7. Unlike MnP and Lac, LiP does not require the addition of mediators such as Mn^{2+} in the oxidation of lignin. Mediators are often added to enhance the oxidation potential of laccase on non-phenolic compounds in delignification of pulp industry and bioremediation of paper mill effluent (Martin-Sampedro et al., 2011, Vivekanand et al., 2008, Ibarra et al., 2006, Minussi et al., 2007a). Due to the uniqueness of LiP in degrading high potential substrate without mediators, it can be applied to treat paper mill effluent and delignification of pulp in place of Lac and MnP systems.

Capability in degrading dye is another added value of this purified LiP which showed activity with both Phenol red and Malachite green at pH 7, although no significant activity was observed at pH 2.7. *Phaneroachete chrysosporium*, *Pleurotus sapidus* and *Trametes trogii* were involved in Phenol Red decolorisation. Phenol red is the common substrate used to detect MnP. Nevertheless, numerous peroxidase such as versatile peroxidase (VP) and LiP also found to be associated with the decolorisation of phenol red (Dass and Reddy, 1990, Schüttmann et al., 2014, Levin et al., 2005). Thus, it is not surprising that purified LiP from *Bacillus licheniformis* shows detectable activity towards phenol red. Apart from phenol red, LiP is able to degrade Malachite green (MG) that has been associated with MnP, laccase, NADH-DCIP reductase and MG-reductase (Du et al., 2013, Du et al., 2011). Although extensive studies have been done on decolorisation of MG by bacteria, no study on degradation of MG with purified bacterial LiP has been reported. To date, this is the

first report on degradation of MG by purified bacterial LiP. Apart from that, Azure B dye was reported as the most suitable test for the ability of LiP in degrading synthetic dye as this dye is hardly degraded by MnP and Lac (Kaur and Sharma, 2022). With the ability of LiP in degrading triarylmethane dye and high redox potential thiazine dye-Azure B, it is conceivable to use LiP in bioremediation of wastewater from textile industry that comprised of dye as the major pollutant.

6.6 pH optimum and stability of LiP

The effect of pH on LiP activity was investigated using substrate VA at pHs ranging from 1.5 – 3.5. As shown in Figure 6.4, the pH profile is skewed towards right with maximum activity at pH 2.0 and no appreciable activity is observed below pH 1.5 and above pH 3.5. This result is in accordance with several fungal LiP that also exhibited narrow working pH for VA oxidation with acidic optimal pH at pH 2.2-3.0 (Yadav et al., 2009, Zeng et al., 2013). Moreover, negligible activity was also shown in fungus *Irpex lacteus* at pH greater than 4.5 (Rothschild et al., 2002). Acidic pH profile was also reported in bacterial LiP which was carried out using other substrates (n-propanol, 2,4-DCP). *Pseudomanas* sp. SUK1 and *Bacillus* VUS showed maximum activity at pH 3.0 and *Acinetobacter calcoaceticus* at pH 1.0 (Kalyani et al., 2011, Dawkar et al., 2009, Ghodake et al., 2009). Similarly, Min et al. (2015) also revealed maximum VA oxidation by DyP from *Bacillus subtilis* at pH 3 (Min et al., 2015).

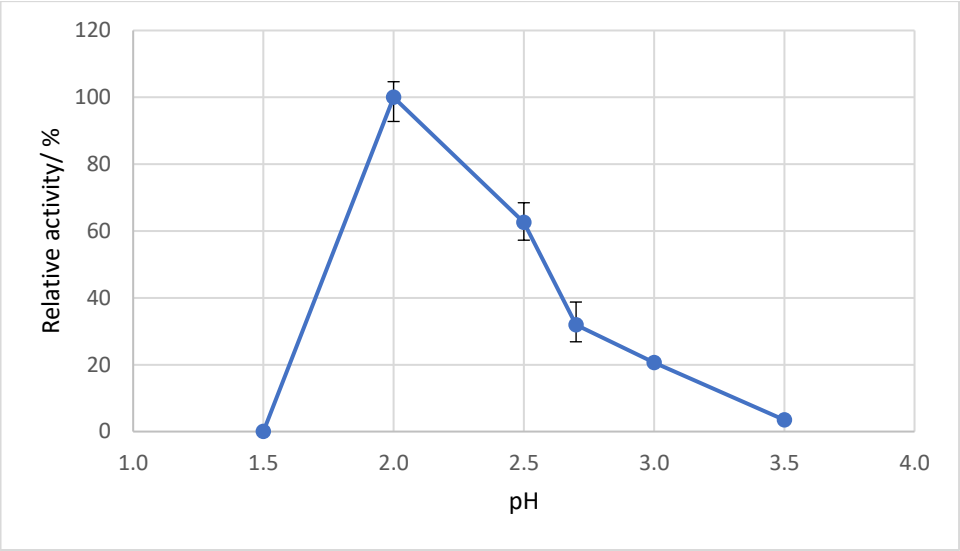

Figure 6.4 Effect of pH on activity of partially purified LiP from Bacillus licheniformis CLMTCHB29 LiP activity of the purified LiP was examined in the pH range of 1.5-3.5 using following buffer system :50mM KCl-HCl buffer (pH 1.0-2.5) and 50 mM citrate phosphate (pH 2.5- 4). LiP activity was assayed under standard assay condition. The activity of the enzyme at pH 2 was taken as 100%. Each point represents the mean result of three replicate with error bar indicating the maximum deviation from the mean value.

As shown in figure 6.5, the LiP exhibits stability over a wide pH range from pH 3.0-9.0 and the enzyme's residual activity recorded for above 50% at pH 3.0 to pH 9.0 after 10 h of incubation. It is most stable at pH 7.0 and pH 9.0 as it retains above 80% of its original activity after 24 hours. The enzyme still has the residual activity of 74% at pH 9.0 even after 48 h of incubation. LiP from *B. licheniformis* shows greater stability than alkali-tolerant DyP from *Saccharomonospora viridis* DSM 43017, which exhibited a decrease of 20% of initial activity after incubation for 1 h in buffers ranging from pH 5 – 10 (Yu et al., 2014). Peroxidase from *Raoultella ornithinolytica* OKOH-1 and *Phanerochaete chrsysosporium* also exhibited lower stability, whereby only 50% of residual activity was retained after incubation of 2 h at pH 6-7 and 1 h at pH 3-6.5 respectively (Falade et al., 2019c, Gu et al., 2003), In contrary, typical peroxidases from *Irpex* lacteus, *Phanerochaete chrysosporium* and *Streptomyces* sp. strain AM2 were found to be highly stable at acidic pH ranging from 3 – 6 with almost no loss of activity after 24 h of incubation at 25 ℃ or 40 ℃ (Salvachúa et al., 2013, Zeng et al., 2013, Fodil et al., 2011).

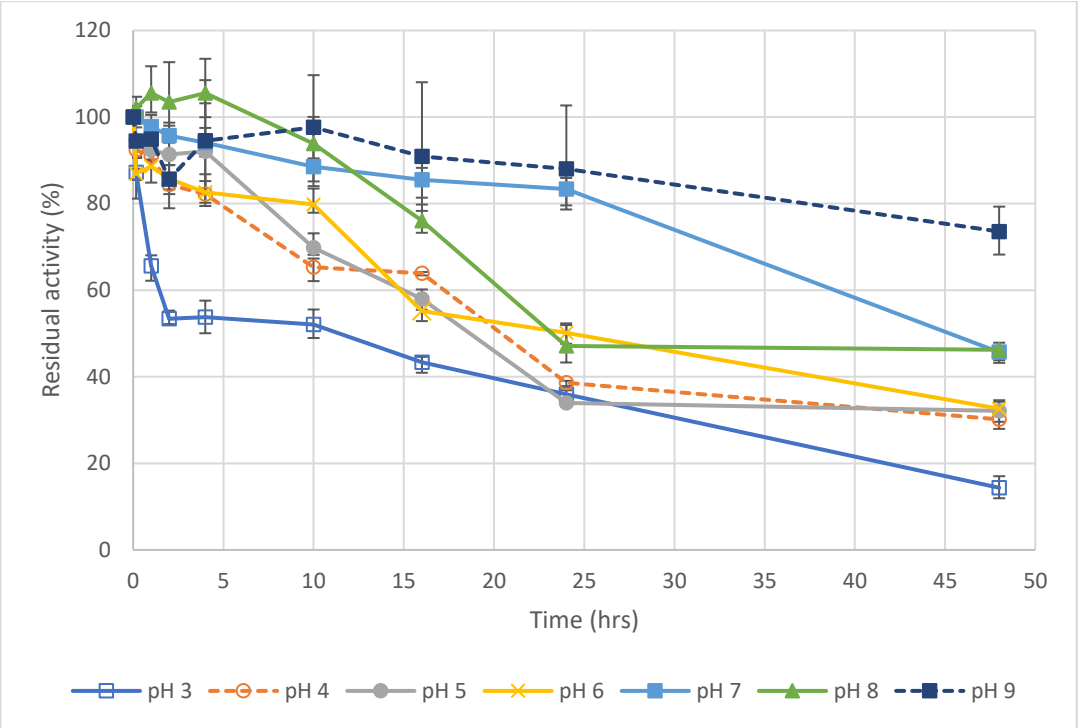

Figure 6.5 Effect of pH on stability of partially purified LiP enzyme from Bacillus licheniformis CLMTCHB29
The thermostability of the LiP was examined by incubating LiP in different buffer system (50mM KCl-HCl buffer (pH 1.0-2.5) and 50 mM citrate phosphate (pH 2.5- 4), 50 mM sodium acetate (pH 4-5) or 50 mM sodium phosphate (pH 6-8) or Tris-HCl buffer (pH9)) ranged from pH 3- 9 for 48 h. Residual activity was assayed under standard assay condition. The activity of the enzyme before incubation was taken as control (100%).

This finding revealed the enzyme has broad pH adaptability particularly at alkali condition. Wide working pH range properties suggests that LiP is capable of oxidising phenolic compound optimally at pH 4-5 (Salvachúa et al., 2013). This is useful in enzymatic hydrolysis of lignocellulose material that work at acidic condition. Apart from that, the alkali tolerant properties of LiP may play an important role in decolorising dye and bioremediation of wastewater. SviDyP from *Saccharomonospora viridis* DSM 43017, Peroxidase from *Serratia* sp. and *Raoultella ornithinolytica* have shown dye decolourisation ability at alkaline region from pH 6-7.6 (Yu et al., 2014, Haq et al., 2016, Falade et al., 2017). Recently, alkali tolerant DyP type peroxidase from from *Serratia* sp. AXJ-M was reported to be effective in degrading lignin content in papermaking black liquor with working pH at 9.53 (An et al., 2021).

Interestingly, it was observed that LiP was stable over neutral to alkali condition, but optimally catalysed VA at acidic region, pH 2. This phenomena is rarely reported but similar pattern was found in DyP from *Pseudomanas aeruginosa* PKE117

with optimum pH was 3.5 and the greater stability in the pH range of 6.5–7.0 when using Reactive Blue 5 as substrate (Li et al., 2012). Study by McEldoon et al. (1995) demonstrated horseradish peroxidase (HRP) is capable to oxidising VA at pH 2.4 but the enzyme appeared to be inactivated quickly under highly acidic condition. In the absorbance study of HRP, increasing soret band A_{372} during the oxidation of VA under acidic condition showed the release of protoporhyrin heme from HRP active site that was inferred to cause the inactivation of the enzyme (McEldoon et al., 1995). Piontek et al. (1993) suggested that the reactivity of peroxidase is related to structure of H-bonding network in axial ligand of the enzyme structure. Axial proximal His of LiP from *Phanerochaete chrysosporium* is made up from Phe with a H-bond between His and Asp, it was predicted this less basic proximal His which may result in higher reactivity. This finding futher supported the observation that LiP is more reactive at low pH (Piontek et al., 1993). However, there is limited study on the structure of LiP that is responsible for reactivity and stability. Thus, further investigation is needed to understand which active site is responsible for reaction and stability of LiP enzyme. The alkaline tolerant properties of LiP gives further support for its potential application in bioremediation of textile wastewater and pulp and paper mill effluent that have existing pH 6 to 9 (Yu et al., 2014, Haq et al., 2016, Ghodake et al., 2009).

6.7 Optimum temperature and thermal stability of LiP

The optimum temperature for LiP was examined at various temperatures between 25℃ and 80℃ and measured using LiP assay at pH 2.7. LiP activity at each tested temperature was illustrated in Figure 6.6. The result revealed that LiP activity exhibits a broad temperature range of 40-70 ℃ with optimal temperature at 50℃. Although there is decline of enzymatic activity was observed below 40℃ or above 70℃, about 79% and 68% of relative activity was maintained at 25℃ and 80℃ respectively. The decrement of LiP activity at 80℃ was likely due to denaturation by heat and changes to the three-dimensional structure of the protein (Kumar et al., 2011).

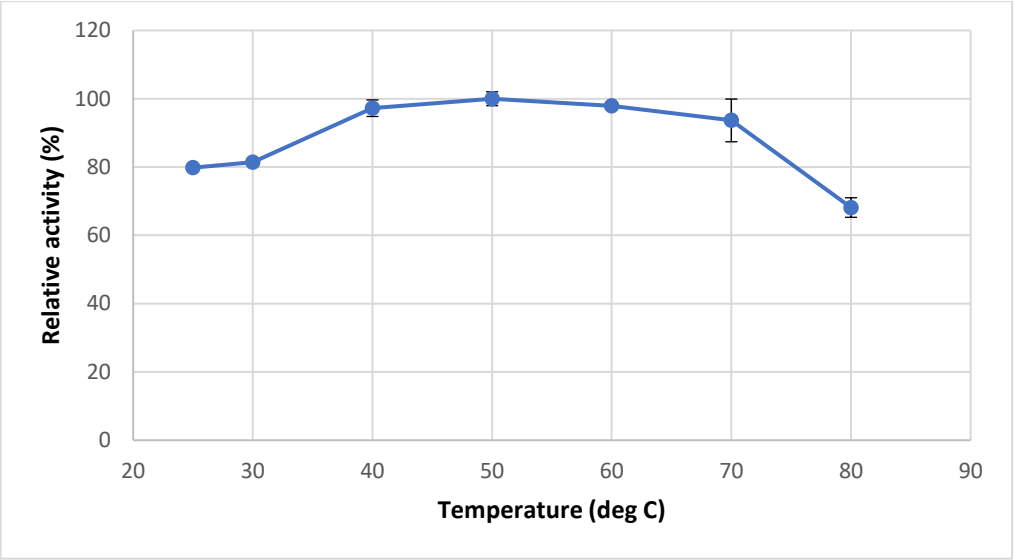

Figure 6.6 Effect of temperature on the activity of partially purified LiP from Bacillus licheniformis CLMTCHB29
The effect of temperature on the activity of LiP was examined at 25-80℃ for 30 min. All measurements were taken at standard assay conditions. The activity of LiP at 50℃ was taken as 100%.

LiP in the current study is considerably more thermotolerant than fungal peroxidase from *Phanerochaete chrysosporium*, *Loweporus lividus* and *Pseudomonas* which reported optimum temperature at relatively low temperature which is 30℃, 24℃ and 40℃ respectively (Ürek and Pazarlioğlu, 2004, Yadav et al., 2009, Kalyani et al., 2011). In fact, the optimum pH of LiP was found comparable with thermotolerant peroxidases from *Kocuria rosea*, *Bacillus subtilis* and *Streptomyces thermovialacues* (Min et al., 2015, Parshetti et al., 2012, Lodha et al., 1991). *Bacillus* sp. VUS was found to work optimally at 65℃ (Dawkar et al., 2009). The properties of broad working temperature range of LiP in the present study are consistent with the studies by Ghodake et al. (2009) and Santos et al. (2014) on LiP from *Acinetobacter calcoaceticus* and DyP from *Bacillus subtilis* (Ghodake et al., 2009, Santos et al., 2014).

The thermostability of the *B. licheniformis* strain CLMTCHB29 LiP was determined by incubating it at temperature ranged from 60- 70℃. The thermal stability profiles (Figure 6.7) revealed that LiP retained about 100% of their initial activity after 24 h incubation at temperature 60℃ to 70℃. Moreover, incubation performed at 70℃ demonstrated that LiP did not show any decrease of activity for 24 hr.

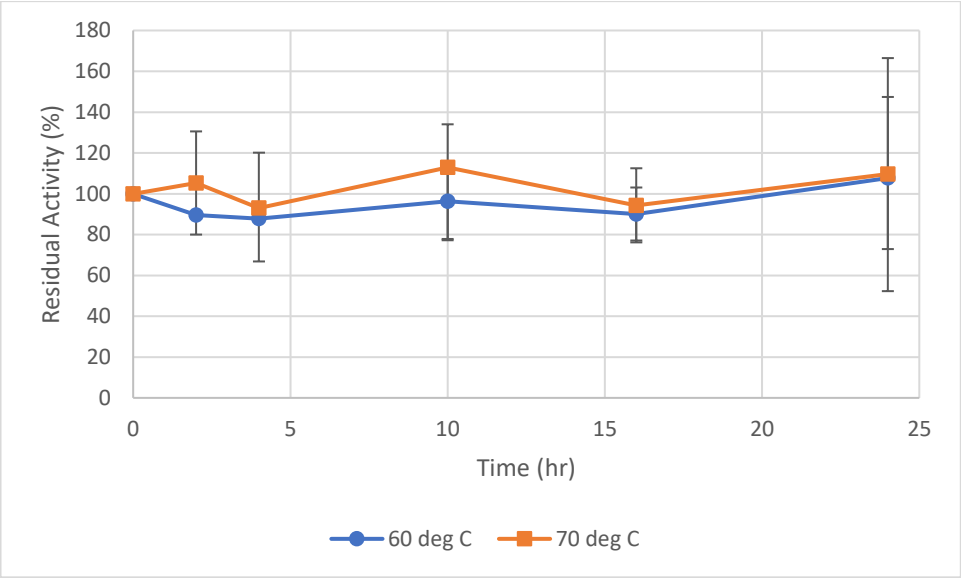

Figure 6.7 Effect of temperature on the stability of partially purified LiP from Bacillus licheniformis CLMTCHB29
The thermostability of the LiP was examined by incubating LiP for 24 h at different temperature (60 and 70℃). The non-heated LiP was considered as 100%. All measurements were assayed under standard assay condition.

Similar thermal stability profile was also reported by Campos et al. (2016) whereby LCC3 laccase enzyme from *Trametes trogii* BAFC 463 showed an increase of up to 30% of relative activity in the first 30 min of incubation and retained 100% of initial activity after 3 h of incubation (Campos et al., 2016). It is worth noting, that LiP in this study seems to have greater thermostability than those thermotolerant peroxidases previously reported in other ligninolytic fungi and bacteria. For instance, Sugawara et al. (2017) and Cai et al. (2010) reported peroxidase inactivated rapidly at temperature greater than 60℃ and 55℃ and exhibited half-life of about 3 h and 8 h at incubation temperature of 50℃ for peroxidase from *Streptomyces avermitilis* and *Rhizoctonia* sp. respectively (Sugawara et al., 2017, Cai et al., 2010). LiP from *Bjerkandera adusta* strain CX-9 and *Phanerochaete chrysosporium* exhibited a half-life of 4 h and 24 h at 50℃(Bouacem et al., 2018, Zeng et al., 2013). Moreover, two thermotolerant peroxidase, RaoPrx and DyP from *Raoultella ornithinolytica* and *Sacchamonospora viridis* DSM 43017 only capable in retaining residual activity of over 70% and 60% respectively after 2 h of incubation at temperature 70℃ (Falade et al., 2019c, Yu et al., 2014).

The thermal tolerant properties observed for the LiP from *Bacillus licheniformis* appeaer to be consistent with the natural habitat of composting process.

It was reported that lignin degradation occurred actively at at thermophilic phase in composting process, thus, it is not surprised that *Bacillus licheniformis's* LiP that was isolated from thermophilic phase of oil palm EFB composting process exhibit greater half-life indicating greater thermal tolerant capacity (Aarti et al., 2015). The thermotolerant properties are known to be attributed to structural characteristics of the enzyme in the aspect of hydrophobicity, hydrogen bonding, salt bridge formation, helical content and thermolabile adaptive amino acid residue (Kumar et al., 2011). Son et al. (2021) have shown disulfide and ionic salt bridges in helix loop of LiPH8 are responsible for stabilising overall structure resistance against thermostability under acidic condition. Enhancement of extra disulfide and ionic bridge have resulted in greater half-life of LiPH8 and expected to facilitate improvement of lignin degradation efficiency (Son et al., 2021). To the best of our knowledge, LiP from the current study have shown greater thermostability properties compared to other LiP from bacteria reported so far. Thus, this thermotolerant LiP has the potential to be further developed for application in the lignin degradation. However, the knowledge on the enzyme structure that is responsible for the thermostability of the LiP is lacking. For practical application of catalytic lignin degradation, it is necessary to understand the association between enzyme structure and LiP thermostability through X-crystallography.

6.8 Effect of metal ions on LiP

The effect of metal ions on LiP activity was examined using VA as the substrate. Table 6.5 illustrate the effect of 1 mM concentration of metal ions on relative LiP activity compared to control sample that is without addition of any metals. The addition of 1 mM of Ca^{2+} and K^{+} had similar activity with control whereas LiP activity. It was observed that the activity of LiP was significantly enhanced in the presence of 1 mM Mn^{2+} and Ni^{2+} followed by Mg^{2+} and Na^{2+} with slight activating effect. According to the result, about 27% and 34% of LiP activity was boosted by Mn^{2+} and Ni^{2+} respectively. Several studies have demonstrated low concentration of Ni has the stabilising and stimulating effect on peroxidase enzymes (Nazari et al., 2007, Tayefi-Nasrabadi et al., 2006). Tayefi-Nasrabadi et al (2006) reported nickel concentration lower than 10 mM could stimulate Horseradish Peroxidase. The stimulating effect could be attributed to the binding of Ni^{2+} to the folded state of HRPC and thus

increasing the accessibility of heme through the stabilization of secondary structure of enzyme whereas Nazari et al. (2007) suggested 2 mM of Ni^{2+} could protect enzyme against suicide-peroxide inactivation. Regarding the effect of Mn^{2+}, the results was consistent with that of Fodil et al. (2011). The Mn^{2+} was required for the strengthening of interaction inside the enzyme molecules and the binding of Mn^{2+} to the autolysis site (Fodil et al., 2011). In contrary, peroxidase from *Bacillus* VUS showed inhibition at 10 mM Mn^{2+} (Dawkar et al., 2009). Similarly, LiP-type activity of POX_Ba enzyme from *Bkerkandera adusta* also inhibited by 2mM of Mn^{2+}.

Table 6.5 Effect of metal ions on activity of partially purified LiP from Bacillus licheniformis CLMTCHB29

Metal ions	**Concentration (mM)**	**Relative activity of LiP[a,b,c] (%)**
Control	-	100±0.0
Fe^{3+}	0.1	11±12.6
	1	0
Mg^{2+}	1	111± 6.7
Fe^{2+}	0.1	28±1.4
	1	0
K^{+}	1	104±12.7
Na^{+}	1	112±13.5
Ca^{2+}	1	97±1.1
Mn^{2+}	1	134±8.3
Ni^{2+}	1	127±9.7

[a] The non-treated enzyme was served as control (100%). The relative activity is expressed as a percentage of activity level in the absence of metal ions.
b The result represents the means of three replicates and standard error are reported.
[c]All measurements were carried under standard assay conditions.

On the contrary, LiP enzyme was highly inhibited by Fe^{2+} and Fe^{3+} whereby no activity was observed at 1 mM for both metal ions. Thus, the effect of Fe^{2+} and Fe^{3+} on LiP activity was further examined at a lower concentration of 0.1mM of Fe^{2+} and Fe^{3+}. The result revealed about 11% and 28% of relative activity for 0.1 mM of Fe^{3+} and Fe^{2+}. The inhibition effect by Fe^{3+} agreed with the previous study on DyP and R-MnP that showed 74.1% and complete inhibition at 5mM and 1mM of Fe^{3+} respectively (Yu et al., 2014, Cai et al., 2010). Similarly, inhibition by Fe^{2+} was also reported in MnP BA30 from *Bjerkandera adusta* strain CX-9, LiP and MnP from *P. chrysosporium*, and MnP from *Trametes polyzona* KU-RNW027 (Bouacem et al.,

2018, Zeng et al., 2013, Lueangjaroenkit et al., 2019). In contrary, Chauhan and Choudhury (2020) reported 4 mM of Fe^{2+} increase LiP activity of *Halopiger aswanensis* (Chauhan and Choudhury, 2020). Poulos et al. (1993) demonstrated Fe^{2+} has the stabilising effect due to the formation of Iron-Histidine bond at active site of LiP that form pocket for hydrogen peroxidase binding and increase its reduction potential leading to increased LiP activity (Poulos et al., 1993). The effect of 1 mM Fe^{2+} on LiP activity seem to vary with those past literature. In fact, Lueangjaroenkit et al. (2019) and Zeng (2013) have demonstrated concentration of metal ions is another concerning factor in affecting the enzyme activity. In Lueangjaroenkit et al. (2019) study, the effect of Fe^{2+}, Ca^{2+}, Mg^{2+} and Mn^{2+} on MnP produced by *Trametes polyzona* KU-RNW027 was investigated. Activation effect of Fe^{2+} on MnP was very narrow compared to other metal ions. Activation effect was observed at concentration range 0.05-0.1 mM and 1- 10 mM for Fe^{2+} and other metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+})

Overall, the result has shown most of the tested metal ions at 1 mM concentration showed positive influence on LiP activity except for Fe^{2+} and Fe^{3+}. This indicated that LiP has certain tolerance towards metal ions. However, concentration of metal ions is another critical factor in affecting the enzyme activity. Thus, it is worth exploring the stability of LiP at various concentration of metal ions and understand the enzyme structure-function relationship in the presence of metal ions since this may offer advantages for implementation of LiP in the treatment of metal contaminated lignocellulose waste (Zeng et al., 2013, Lueangjaroenkit et al., 2019).

6.9 Effect of inhibitors on LiP

The result on effect of 0.1 mM of inhibitors on LiP activity in relative to control was illustrated in Table 6.6. It showed that the presence of 0.1 mM EDTA and Tween 80 in the reaction mixture has stimulating effect on LiP enzyme activity with residual activity of 105% and 102%. However, addition of 0.1 mM of SDS and NaN_3 to the reaction mixture resulted in inhibition of LiP activity with residual activity of 78% and 89% respectively.

Table 6.6 Effect of inhibitors on the activity of partially purified LiP from Bacillus licheniformis CLMTCHB29

Inhibitors	Concentration (mM)	Relative activity of LiP[a,b,c] (%)
SDS	0.1	100±2.3
EDTA	0.1	78±4.3
Tween 80	0.1	105±15.0
NaN_3	0.1	102±1.5

[a] *The non-treated enzyme was served as control (100%). The relative activity is expressed as a percentage of activity level in the absence of inhibitors.*
b The result represents the means of three replicates and standard error are reported.
[c]*All measurements were carried under standard assay conditions.*

The finding is consistent with previous studies, where significant inhibitory effect on peroxidase by known peroxidase inhibitor, NaN_3 was reported (Ramachandra et al., 1988, Fodil et al., 2011, Falade et al., 2019c). In fact, LiP enzyme in the current study showed high inhibition by NaN_3 even at the very low concentration at 0.1 mM comparing to DyP that still showed stimulating effect at 1 mM NaN_3 and inhibition was only observed at 5mM NaN_3 (Falade et al., 2019c). NaN_3 is a heme specific reagent, thus, inhibition of LiP activity by NaN_3 indicated the presence of heme components in the tertiary structure of this enzyme (Fodil et al., 2012, Rob et al., 1997).

Previous studies showed that surfactants such as SDS and Tween 80 have significant effect on hydrophobic effect of protein. This probably attributed to the interaction between surfactants and some surface amino acid residues that affect the binding of the substrate at the peroxidase active site (Rekik et al., 2019). Several peroxidases from fungal and bacteria species such as LiP BA45, MnPT55 and LiP-SN from *Bjerkandera adusta* strain CX-9, *Trametes pubescens* strain i8 and *Streptomyces griseosporeus* SN9 showed less inhibitory effect with SDS respectively (Bouacem et al., 2018, Rekik et al., 2019, Rekik et al., 2015); in contrast, DyP from Svi showed complete inhibition at 5 mM of SDS (Yu et al., 2014). Study on effect of Tween 80 on bacterial LiP is scarce, most studies focused on MnP and DyP. Tween 80 promotes activity in MnP from White Rot Fungi such as *Phanerochaete chrysosporium* and *Pestalotiopsis* sp. NG007 (Hirai et al., 2004, Yanto et al., 2014). However, inhibition was observed on DyP from *Nonomuraea gerenzanensis* (Casciello et al., 2017).

Chapter 7 Conclusion and Recommendation

7.1 Conclusion

Numerous isolates that were successfully screened from EFB compost in this study indicated that thermophilic ligninolytic bacteria are actively involved in the decomposition of EFB. The isolates from Empty Fruit bunch exhibited different decolourisation ability compared to those conventional lignin degrading fungi in solid plate assay containing Methylene Blue and guaiacol. Thus, the screening plate assay method that was initiated for the lignin degrading fungi might not be necessarily suitable for lignin degrading bacteria. The isolates are capable producing LiP, MnP and Lac whereby LiP is the predominant enzyme. It was identified that LiP and MnP produced in the first 24 h whereas Lac production starts increasing toward the end of fermentation process.

Isolate CLMT29 can produce three main ligninolytic enzymes namely LiP, MnP and Lac. This strain was selected for further optimisation and characterisation study since it expressed the highest titre of LiP among the tested isolates. By comparing the sequence of the 16S rRNA gene with the database in GenBank using BLASTn tool and phylogenetic tree confirmed that the strain CLMT 29 was closely related to *Bacillus licheniformis*. It was deposited in GenBank Database named as *B. licheniformis* CLMTCHB29 with Accession number MH 197076.

In Chapter 5 One factor at a Time Approach was employed to study the effect of various nutritional and physical parameters on LiP production, determine the efficient range of each parameter while maximising LiP production. It was found that LiP was only expressed by *Bacillus licheniformis* CLMTCHB29 under submerged fermentation at optimal temperature of 50 ℃, neutral pH, with 16 % (v/v) inoculum size and at relatively short incubation time of 15 h (late logarithm growth phase). The optimal nutritional conditions were observed at 0.4 % (w/v) glucose, 0.2% (w/v) yeast extract, 0.08% (w/v) kraft lignin and 0.06% (w/v) $CaCl_2$ respectively. Following optimisation study, the application of optimal conditions resulted in 40% of increment in the LiP production from 10 U/L to 14 U/L.

The finding showed that secretion and production of LiP is closely associated with the active growth of the bacterial life cycle. Optimal growth at 50 ℃ inferred that *Bacillus licheniformis* is thermophilic and its LiP exhibits thermotolerant properties. Moreover, LiP production is favoured at pH greater than pH 7 indicating enzyme is alkaliphilic. Based on the nutritional study on glucose and lignin, LiP was expressed in the absence of glucose or lignin whereas addition of 0.4% (w/v) glucose and 0.06% (w/v) lignin stimulated LiP production by about 3 and 3.5-fold respectively. Most ligninolytic microorganisms only expressed LiP in the presence of both glucose and lignin. This newly isolated strain of *Bacillus licheniformis* is capable of utilising kraft lignin as sole carbon source whereby most of the ligninolytic microorganisms require glucose as secondary carbon source. Apart from that, yeast extract is critical in LiP production since no LiP was expressed in medium without yeast extract. Yeast extract could be involved in NADH regeneration which acts as electron donor in metabolic pathways of microbes and aids in peroxidase production, However, yeast extract greater than 0.2% (w/v) inhibited the LiP production that may be due to high accumulation of toxic by-products during fermentation process. Calcium concentration of less than 5.4 mM showed stimulation effect on the LiP production, and this might be attributed to the requirement of calcium in maintaining the integrity of LiP active site. Supplementation of calcium has been shown to stimulate cell growth and affect the cell morphology and favours the uptake of nutrients which further leads to greater enzyme production (Knežević et al., 2018, Pazla et al., 2020).

However, little is known about the characteristics of LiP from *Bacillus licheniformis*. Characteristics and stability of an enzyme are important aspects in examining the potential of enzyme in lignin degradation application. Thus, in chapter 6, partially purified LiP was isolated through ultrafiltration using Amicon stirred cell paired with Ultra regenerated cellulose 10 kDa membrane followed by Bio-Gel P-100 gel chromatography purification process. After the two steps purification process, 2.08% of protein was recovered with purification fold of 0.60. Low purification fold of LiP observed could be attributed to the inefficiency of Ultra regenerated cellulose 10 kDa membrane in retaining 26 kDa LiP enzyme and loss of enzyme activity during the purification process. It was reported that 64.8% of LiP enzyme remained in the permeate of ultrafiltration and 26.6% loss in enzyme activity during the ultrafiltration process. The partially purified LiP showed a single homogeneous band at 26 kDa on

SDS-PAGE gel with pI value of 1.56 that was determined using Zeta potential technique. The partially purified LiP has maximum activity at pH 2.0 and temperature 50℃with 0.76 mM and 3.78 U mg^{-1} of Km and Vmax values, when veratryl alcohol was used as a substrate. Apart from that, Km and Vmax values with respect to substrate H_2O_2 were reported as 80 mM and 15.06 U mg^{-1}. The substrate specificity activity among the tested substrate in relative to veratryl alcohol was in the following order: 2-6, DMP > ABTS>Guaiacol> VA> Malachite Green> Azure B, but no activity was observed in phenol red.

In terms of enzyme's pH stability, the enzyme recorded residual activity of at least 50% at pH 3.0 to pH 9.0 after 10 h of incubation time at room temperature. Surprisingly, LiP was also reported to retain about 100% of residual activity after incubated for 24 h at temperature 60 ℃ to 70 ℃. This is the first report on thermotolerant LiP isolated from *Bacillus licheniformis* CLMTCHB29. Activity was highly inhibited by Fe^{3+} and Fe^{2+} even at low concentration of 0.1 mM. In the presence of 1 mM Mn^{2+} and Ni^{2+}, the residual activity of LiP was boosted to 134% and 127% respectively, whereas 1mM of Na^{2+} and Mg^{2+} stimulated LiP activity to about 112% ad 111% respectively. The common peroxidase inhibitors such as SDS and NaN_3 recorded residual activity of 78% and 89% at 0.1 mM final concentration. Whereas no significant effect on LiP activity was observed on EDTA and Tween 80. It is inferred that metals ion play an important role in maintaining the stability and activity of LiP. Thus, it is worth to explore role of metals in term of its catalysis and functionality of LiP. The thermotolerant, slight alkaliphilic and metal tolerance properties demonstrated that LiP may be potentially useful in the degradation of high temperature, alkaliphilic and metal contaminated lignocellulose waste.

7.2 Limitation and Recommendation

The work presented in this thesis confirmed the presence of thermophilic lignin degrading bacteria in Empty Fruit Bunch and its potential in degrading kraft lignin using *Bacillus licheniformis*. Moreover, this study also provides some understanding on production and characterisation of LiP from *Bacillus licheniformis* strain CLMTCHB29 that could be used as a reference in LiP application. Although this thesis has provided a significant contribution above, below are some recommendations for future study.

1. Based on the FTIR analysis, changes on the functional group of lignin structure indicate *Bacillus licheniformis* CLMTCHB29 is capable in degrading lignin. Considering the significant catalytic efficiency of *Bacillus licheniformis* CLMTCHB29 on lignin degradation, a detailed understanding on the role of LiP and its mechanism on lignin or lignin derivative degradation at molecular level is worth pursuing in order to develop an effective lignin enzyme degradation system.
2. In this study, metal ions such as Mn^{2+}, Ni^{2+}, Na^{2+} and Mg^{2+} demonstrated activating effect on LiP enzyme whereas Fe^{2+} and Fe^{3+} inhibited LiP activity. There is a report that showed metals ions may act as activator/ inhibitor in coordinating to active site residues leading to activation or inhibition of enzyme and thus affecting the affinity of enzyme and substrate. However, knowledge is still lacking on the activation mechanism of metal ions on the active site residue of this newly isolated LiP enzyme. Increasing catalytic/degradation efficiency is always the critical aspect for practical industrial used. Thus, it is suggested to carry out in-depth study on the role of metal ions on the affinity of enzyme and substrate to enhance the performance of LiP.

3. The LiP enzyme from *Bacillus licheniformis* CLMTCHB29 exhibited thermotolerant and alkaliphilic properties. Literature reported these extremophilic properties might be correlated to structural properties of the enzyme in the aspect of hydrophobicity, hydrogen bonding, salt bridge formation, helical content and thermolabile adaptive amino acid residue. However, there is a lack of study on the enzyme structure of this newly isolated LiP. Hence, further study on the enzyme structure that is responsible for thermal stability and alkali properties is recommended. A deeper understanding on the mechanism that confers thermal stability and alkaliphilic properties of LiP would be useful in developing the capacity of LiP for industrial application.

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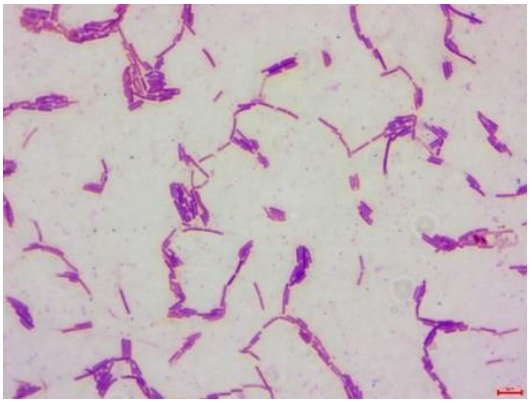
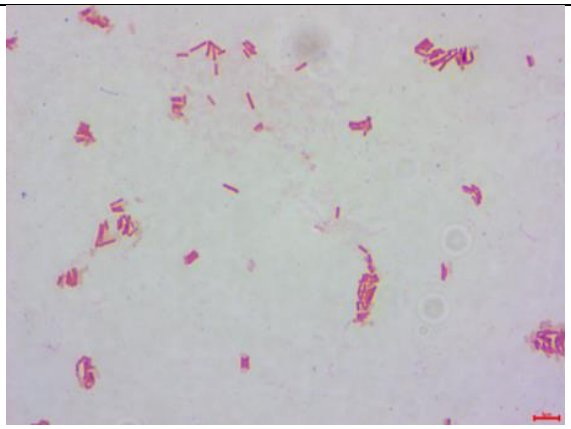
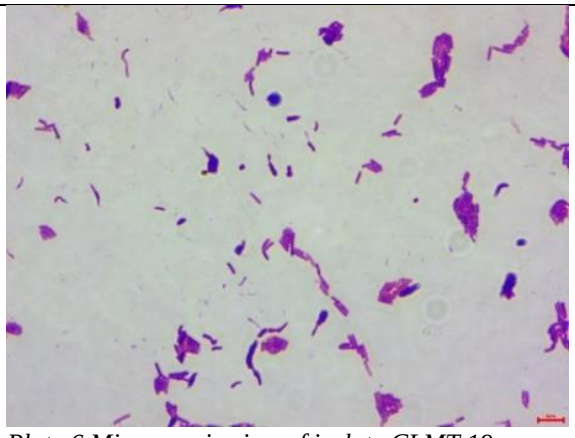
Appendix A Agar plate and microscopic view of Isolates

Colonies	Microscopic view
 *Plate 1 Isolate CLMT 5 on TSA*	 *Plate 2 Microscopic view of isolate CLMT 5*
 *Plate 3 Isolate CLMT 18 on TSA*	 *Plate 4 Microscopic view of isolate CLMT 18*
 *Plate 5 Isolate CLMT 19 on TSA*	 *Plate 6 Microscopic view of isolate CLMT 19*

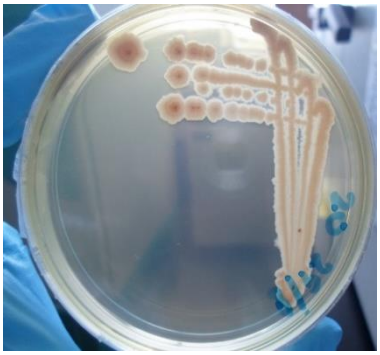


Plate 7 Isolate CLMT 20 on TSA

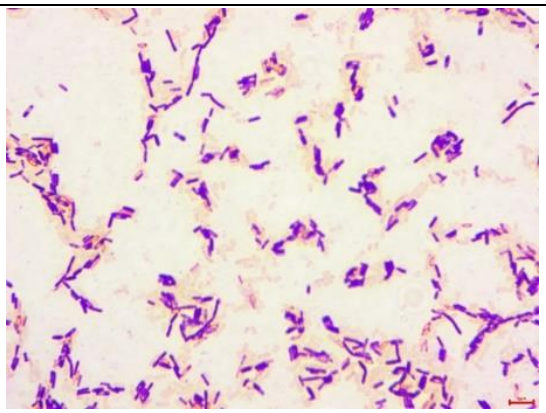


Plate 8 Microscopic view of isolate CLMT 20

Plate 9 Isolate CLMT 21 on TSA

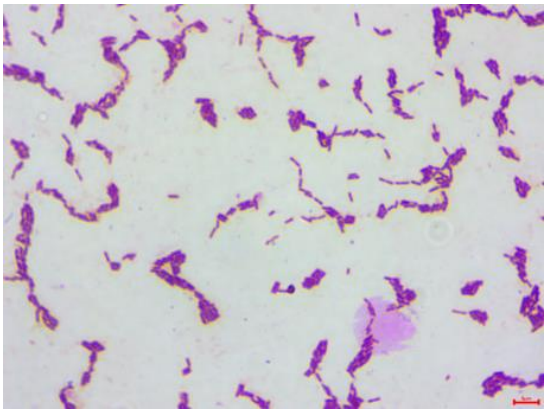


Plate 10 Microscopic view of isolate CLMT 21

Plate 11 Isolate CLMT 22 on TSA

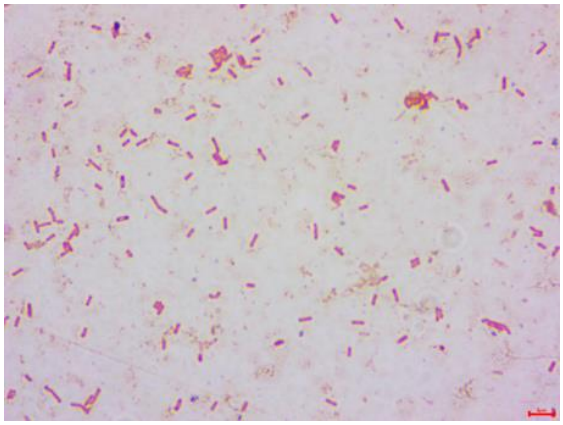


Plate 12 Microscopic view of isolate CLMT 22

Plate 13 Isolate CLMT 23 on TSA

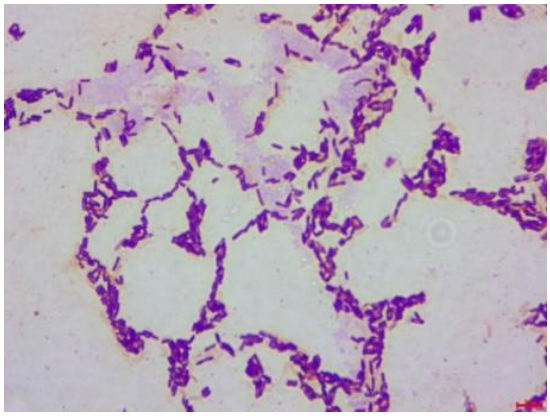


Plate 14 Microscopic view of isolate CLMT 23

Plate 15 Isolate CLMT 28 on TSA

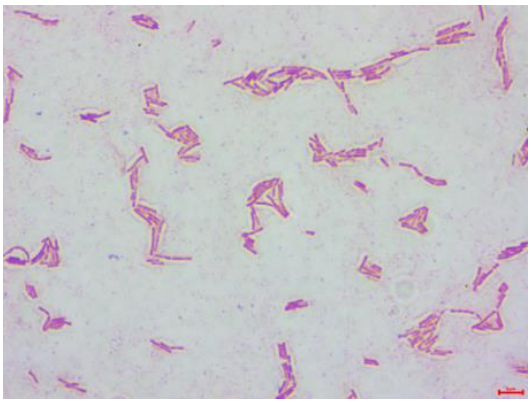


Plate 16 Microscopic view of isolate CLMT 28

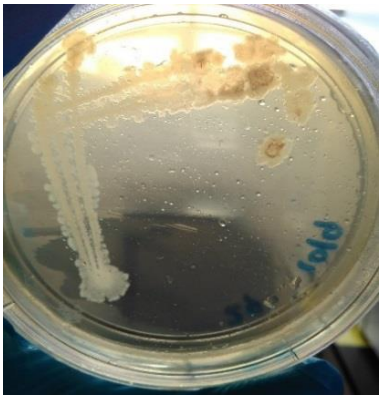


Plate 17 Isolate CLMT 29 on TSA

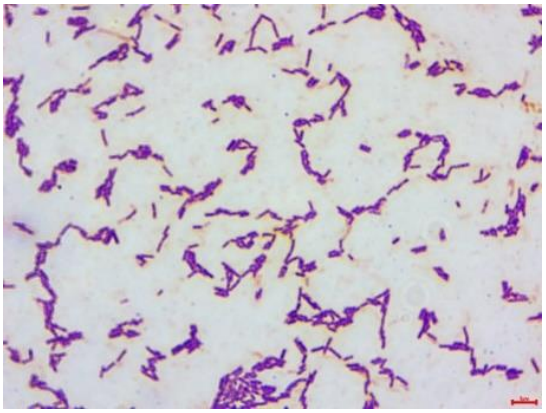


Plate 18 Microscopic view of isolate CLMT 29

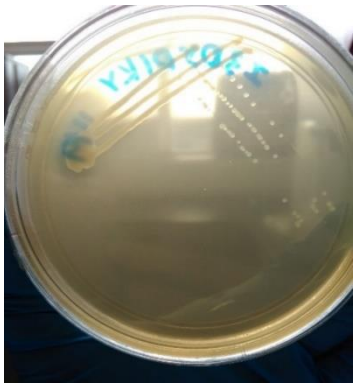


Plate 19 Isolate CLMT 34 on TSA

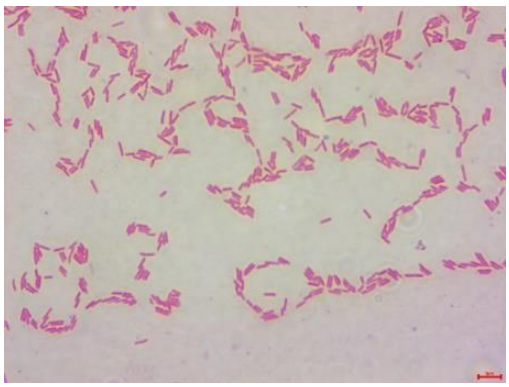


Plate 20 Microscopic view of isolate CLMT 34

Appendix B Growth Profile of *B. licheniformis* CLMTCHB29 in TSB medium

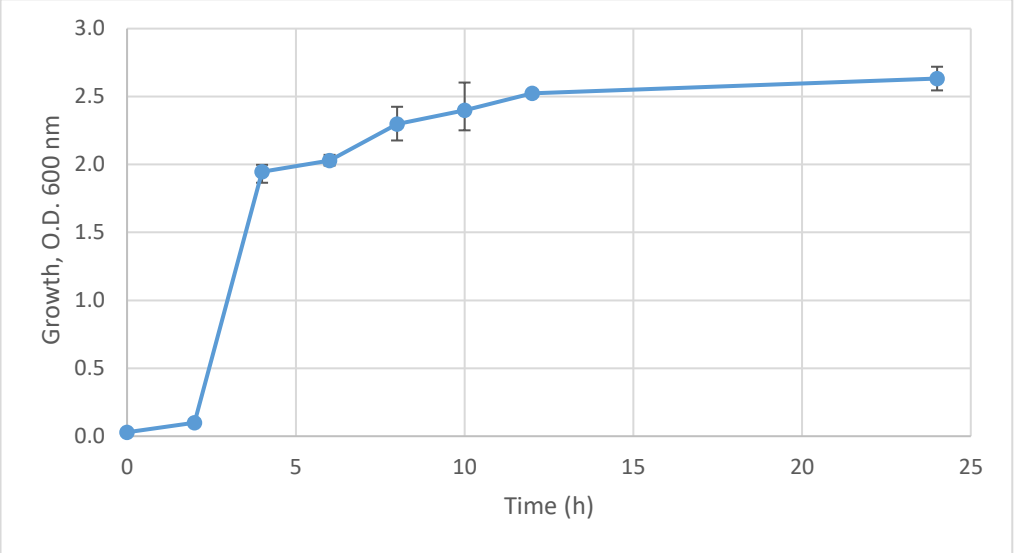


Figure B. 1 Growth profile of B. licheniformis incubated in TSB medium (pH 7) under shaking condition (120 rpm) for 24 h at 50 ℃

Appendix C ANOVA

Table C. 1 ANOVA analysis on LiP production with varying incubation time

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	0.0000	0.0000	0.0000
5	3	0.0000	0.0000	0.0000
10	3	25.2607	8.4202	0.0519
15	3	29.4778	9.8259	0.2008
20	3	26.2125	8.7375	0.1287
25	3	19.9874	6.6625	0.4222

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	298.514	5	59.7028	445.7606	3.43E-13	3.1059
Within Groups	1.6072	12	0.1339			
Total	300.121	17				

Table C. 2 ANOVA analysis on LiP production with varying incubation temperature

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
45	3	22.2600	7.4200	0.0268
47.5	3	22.8200	7.6067	0.0342
50	3	28.5990	9.5330	0.3544
52.5	3	24.5600	8.1867	0.4409
55	3	0.0000	0.0000	0.0000

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	169.0587	4	42.2647	246.7683	6.04E-10	3.4780
Within Groups	1.7127	10	0.1713			
Total	170.771	14				

Table C. 3 ANOVA analysis on LiP production with varying inoculum size

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
4	3	16.9810	5.6603	0.1390
8	3	16.7217	5.5739	0.1904
12	3	19.9277	6.6426	0.0005
16	3	26.5200	8.8400	0.1011
20	3	25.9700	8.6567	0.4372

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	30.1754	4	7.5439	43.4401	2.73E-06	3.4780
Within Groups	1.7366	10	0.1737			
Total	31.9121	14				

Table C. 4 ANOVA analysis on LiP production with varying pH

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
6	3	0.0000	0.0000	0.0000
7	3	27.7183	9.2394	0.3794
8	3	25.6458	8.5486	0.3113
9	3	14.7388	4.9129	0.6104
10	3	0.0000	0.0000	0.0000

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	238.5475	4	59.6369	229.1811	8.7E-10	3.4780
Within Groups	2.6022	10	0.2602			
Total	241.1497	14				

Table C. 5 ANOVA analysis on LiP production with varying glucose concentration

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	10.2658	3.4219	0.0094
0.2	3	33.4738	11.1579	4.0029
0.4	3	37.8724	12.6241	0.7275
0.6	3	33.4291	11.1430	0.3687
0.8	3	25.4520	8.4840	1.4542

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	159.2825	4	39.8206	30.3385	1.44E-05	3.4780
Within Groups	13.1254	10	1.3125			
Total	172.4079	14				

Table C. 6 ANOVA analysis on LiP production with varying yeast concentration

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	0.0000	0.0000	0.0000
0.2	3	40.3300	13.4433	2.3376
0.4	3	27.2701	9.0900	0.4802
0.6	3	17.6679	5.8893	0.6729
0.8	3	20.2837	6.7612	1.7908

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	288.5061	4	72.1265	68.2823	3.2E-07	3.4780
Within Groups	10.5630	10	1.0563			
Total	299.0690	14				

Table C. 7 ANOVA analysis on LiP production with varying lignin concentration

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
0	3	14.4333	4.8111	0.0601
0.02	3	16.0138	5.3379	0.0909
0.04	3	20.8298	6.9433	0.1468
0.06	3	42.6139	14.2046	2.4954
0.08	3	43.7471	14.5824	0.6209
0.1	3	33.0713	11.0238	0.4807

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	288.3898	5	57.6780	88.8516	4.69E-09	3.1059
Within Groups	7.7898	12	0.6491			
Total	296.1796	17				

Table C. 8 ANOVA analysis on LiP production with varying $CaCl_2$ concentration

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1.36	3	25.8546	8.6182	0.1076
2.72	3	27.7557	9.2519	0.0161
4.08	3	42.8972	14.2991	0.1389
5.44	3	36.3665	12.1222	0.6245
6.80	3	29.1815	9.7272	0.0946

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	66.8979	4	16.7245	85.1825	1.1E-07	3.4780
Within Groups	1.9634	10	0.1963			
Total	68.8612	14				

Appendix D Michaelis-Menten and Lineweaver Burk plot

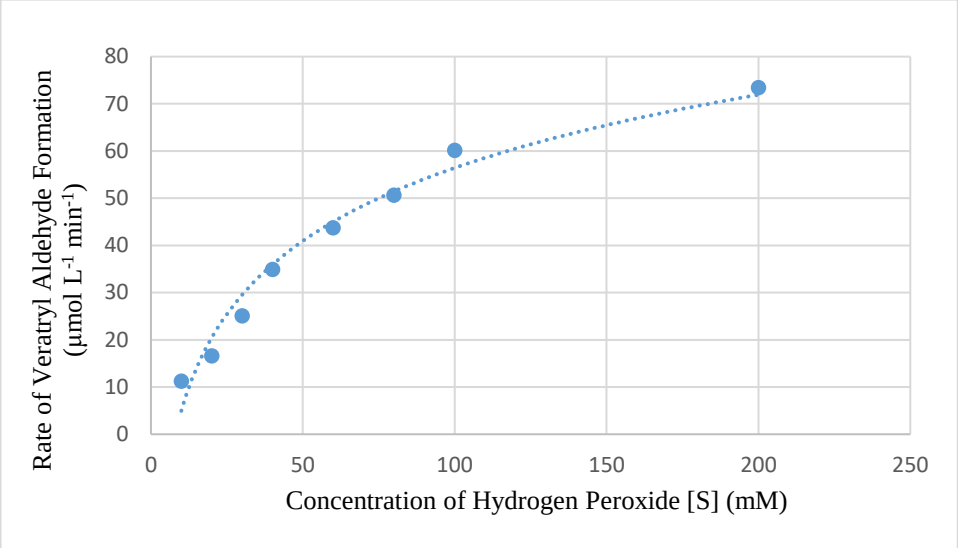


Figure D. 1 Michaelis Menten saturation curve for LiP with varying H_2O_2 concentration at constant VA concentration

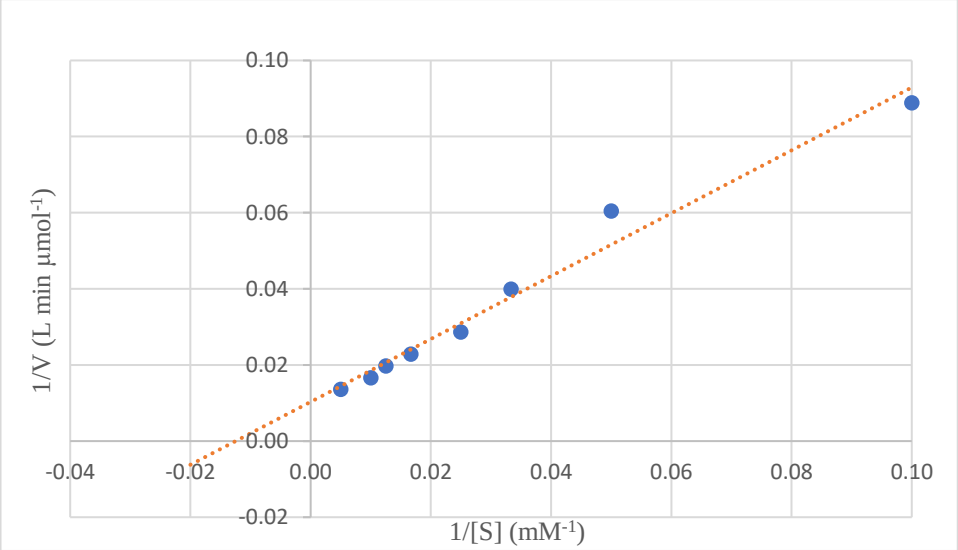


Figure D. 2 Lineweaver Burk plot of H_2O_2 on LiP using VA as substrate

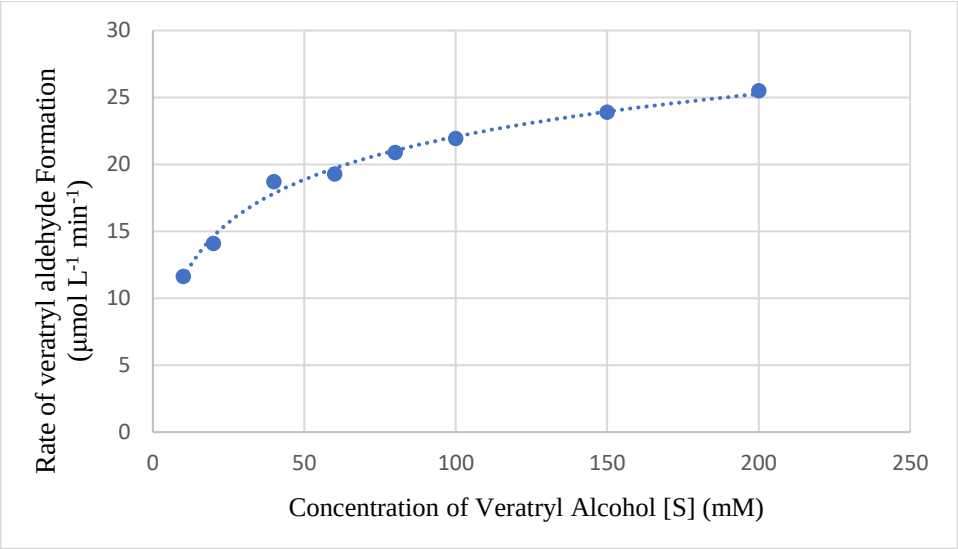


Figure D. 3 Michaelis Menten saturation curve for LiP with varying VA concentration at constant H_2O_2 concentration

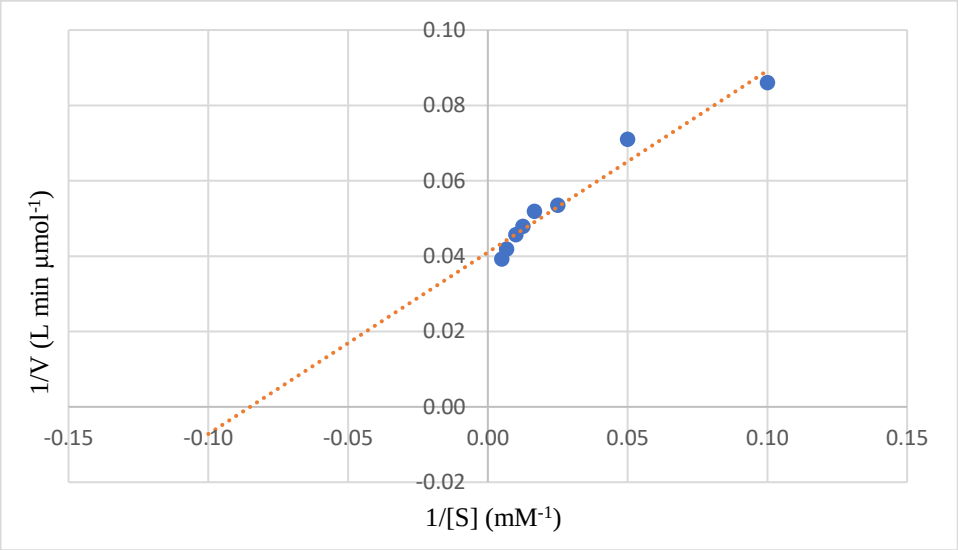


Figure D. 4 Lineweaver Burk plot of veratryl alcohol on LiP

Appendix E Attribution Statement

I declare that below is indication of percentage contribution by each author in each field of activity.

	Conception and Design	**Acquisition of Data and Method**	**Data Conditioning and Manipulation**	**Analysis and Statistical Method**	**Interpretation and Discussion**	**Total % Contribution**
Author (Carol Lai Mei Teng)	50	50	50	50	50	50
Author 1 Acknowledgement: I acknowledge that these represent my contribution to the above research output Signed: [redacted] 21/06/2022						
Co-Author 1 (A/P Chua Han Bing)	20	20	20	20	20	20
Co-Author 1 Acknowledgement: I acknowledge that these represent my contribution to the above research output Signed: [redacted] 20/06/2022						
Co-Author 2 (Prof. Agus Saptoro)	10	10	10	10	10	10
Co-Author 2 Acknowledgement: I acknowledge that these represent my contribution to the above research output Signed: [redacted] 21/06/2022						
Co-Author 3	10	10	10	10	10	10

(Prof. Michael Danquah)						
Co-Author 3 Acknowledgement: I acknowledge that these represent my contribution to the above research output Signed [redacted] 19 June 2022						
Total %	100	100	100	100	100	100

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