JUDUL **SCREENING OF LIGNIN DEGRADER FROM SOIL**

SESI PENGAJIAN: **2007/2008**

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SUPERVISOR'S DECLARATION

"I hereby declare that I have read this thesis and in my opinion this thesis is sufficient in terms of scope and quality for the award of degree in Bachelor of Chemical Engineering (Biotechnology)"

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Date : 16 May 2008

SCREENING OF LIGNIN DEGRADER FROM SOIL

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A thesis submitted in fulfillment of the requirements for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

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MAY 2008

STUDENT'S DECLARATION

"I declare that this thesis entitled Screening of Linin Degrader from Soil is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

Signature :……..
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Date : 16 May 2008

DEDICATION

Specially dedicated to my beloved family, my brothers and sisters from Senandika team, my friends and all of faculty members.

Thank you for your endless support.

Sincerely,

Noor Zanida binti Zamani,2008

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ABSTRACT

Since many years ago many researches have been done in order to find the latest, cheaper and more effective ways of getting out of lignin so that they can get to the cellulose fibers. Lignin is the main problems in the pulps and paper industries. The presence of lignin makes the newsprint turn yellow. Besides that, the presence of lignin also contributed to problems such as acid gives off as the woods deteriorates. So that, in order to produce fine papers it is needed to removes the lignin. Biodegradation of lignin by microorganisms is the latest technology being found to replace the conventional method which using chemicals to remove lignin. Using microorganisms to removes lignin contributed in reducing cost and besides that, this method is really friendly to the environment. The main objectives of this study are to screen, to characterized microorganisms from soil that produce the lignin degrading enzymes and to analyze the enzyme activity. This process includes of two phases: screening process and the characterization process. Screening process was done using selective agar containing alkaline lignin. The microbe was characterized using simple staining, gram staining, and spore staining. The lignin degrading microbe found was tested; the result was a gram positive and, having endospore. The optimum pH was found to be in pH 7.0 while the optimum temperature discovered was 50°C with enzyme activity at 588.3 μmol/ml/min.

ABSTRAK

Sejak beberapa tahun lalu pelbagai kajian telah dibuat dalam mencari jalan dan kaedah terbaru, yang paling murah dan lebih efektif untuk mengeluarkan lignin. Lignin merupakan masalah utama dalam industri pulpa dan pembuatan kertas. Kehadiran lignin menyebabkan kertas yang dihasilkan berwarna kuning. Selain itu, kehadiran lignin juga menyumbang kepada masalah seperti perembesan asid apabila kayu mereput. Oleh sebab itu, dalam menghasilkan kertas yang bagus dan berkualiti, adalah perlu supaya lignin yang terdapat dalam tumbuhan berkayu dibuang terlebih dahulu sebelum diproses menjadi kertas. Biodegradasi lignin oleh mikroorganisma merupakan teknologi terbaru dijumpai bagi menggantikan kaedah lama pembuangan lignin di mana kaedah lama menggunakan bahan kimia yang merbahaya kepada manusia dan persekitaran. Dengan menggunakan bacteria pengurai lignin boleh mengurangkan kos di samping kaedah ini mesra alam dan tidak mendatangkan bahaya kepada persekitaran. Objektif utama kajian ini adalah untuk mengkaji pengskrinan dan pengelasan ciri ke atas bakteria pengurai lignin dan pengelasan ciri ke atas enzim yang dihasilkan oleh bakteria pengurai lignin. Ia melibatkan dua fasa: proses pengskrinan, proses pengelasan ciri. Pengskrinan dibuat dengan menggunakan agar pemilih yang hanya mengandungi alkali lignin sebagai sumber karbon. Bakteria yang dijumpai diuji dengan staining ringkas, gram staining, dan spora staining untuk mengetahui ciri-cirinya. Hasilnya, bakteria yang dijumpai merupakan bakteria gram positif dan mempunyai endospore. pH optima didapati adalah pH 7.0 manakala suhu optima yang didapati adalah 50°C pada aktiviti enzim bersamaan dengan 588.3 μmol/ml/min.

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LISTS OF SYMBOLS/ABBREVIATIONS

ABS	-	Absorbance
BOD	-	Biological Oxygen Demand
COD	-	Chemical Oxygen Demand
cm	-	centimeters
DNS	-	Dinitrosalicyclic colorimetric
g	-	Gram
g/l	-	Gram per liter
hr	-	Hour
kD	-	kiloDalton
min	-	Minutes
mg/ml	-	Miligram per mililiter
ml	-	Mililiter
NA	-	Not Available
nm	-	Nanometer
OD	-	Optical Density
rpm	-	Rotation per minute
sec	-	Seconds
sp.	-	Species
U	-	Unit (enzyme activity)
v/v	-	Volume per volume
w/v	-	Weight per volume
µmol/ml/min	-	Micromol per mililiterper minute
µl	-	Microliter
°C	-	Degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Introduction and Problem Statement

Screening of lignin degrader from soil is all about searching microorganisms which produce enzyme that degrades lignin. Lignin is a substance in trees that hold cellulose fibers together. Lignin is found in all vascular plants and it functions are to regulate transport of liquid in the plant and to reinforce the cell walls in order to avoid them from collapsing. Besides, the advantage of lignin is it can limit the spread of pathogens in plant tissues. However, the advantages are only useful to the plants but not to the industry especially to the paper industries. Lignin will be removed in order to produce fine papers. The present of lignin will contribute problems such as acid gives off as the woods deteriorates. The same problem goes to paper and board which contain lignin.

The structure of lignin is complex. It makes the cellulose and hemicellulose in wood indigestible. It contains benzene ring with a tail of three carbons. It also appears to have a lot of internal H bonds making them very resistant to degradation process naturally (McCrady, 1991). Before being produced as papers, the timber will undergo certain treatments to remove lignin. Conventionally, the paper industries are using the cooking method in order to remove lignin. This method is limited to certain time and has to be stopped before all the lignin can be removed. This is because the cellulose would be gone too if the treatment is continued. To overcome this, they use bleaching method which will remove the remaining lignin. Bleaching method needs

the usage of toxic chemicals such as chlorine. In order to get benefit for both industry and environment, many studies have been done.

Lignin is one of the most abundant natural polymers on earth. White rot fungi are primarily responsible for initiating the depolymerization of lignin in wood. Types of lignin degrading enzyme that can be found are such as lignin peroxidase, manganese peroxidase, glyoxal oxidase and laccase (Howard *et al.*, 2003). Most of these enzyme produced by fungi. Such bacteria that can produce lignin peroxidase is *Streptomyces viridosporus* (Ramachandra *et al.*, 1987). Some previous studies found that *Phanerochaete chrysosporium* also produce lignin degraders enzyme. The chemical and physical properties of this fungus are that they grow and degrade lignin rapidly. Besides, they also have relatively high temperature optimum and they produce no lactase.

A new approach for degrading the lignin has been found in 1982 (Kirk *et al.*, 1985) where they use the enzyme collected from fungi. But due to the fact that fungi are hazardous, other methods in producing the lignin degrading enzymes was found in bacteria. The bacteria will be cultivated to extract the enzymes needed and then being produced in a large quantity for industrial purpose.

1.2 Objective of the study

The objective of the study is to screen and to characterize microorganism from soil that produce the lignin degrading enzymes.

1.3 Scope of the study

The scope of this study consists of two parts which are:

i) Screening of microorganisms from sample soils and
ii) Characterization of the microorganisms and enzymes using morphology tests and enzyme analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignocellulose

Lignocellulose can be defined as a combination of lignin and cellulose that strengthens the woody plants (Freedictionary.com). lignocellulose is the major structural component of woody plants and non-woody plants such as grass and represents a major source of renewable organic matter (Howard *et al.,* 2003).

Lignocellulose consists of lignin, hemicellulose and cellulose and table 2.1 shows the typical compositions of the three components in various lignocellulosic materials (Betts *et al.,* 1991). They are the building blocks of all plants and are the ubiquitous to most regions on earth.

The chemical properties of lignocellulose make them a substrate of enormous biotechnological value. The basic chemical structure of these three compounds in lignocellulose has a profound on lignocellulose tertiary architecture (Malherbe *et al.,* 2002).

Table 2.1: Lignocellulose contents of common agricultures waste and residues (Howard *et al.*, 2003)

Lignocellulosic materials	**Cellulose (%)**	**Hemicellulose (%)**	**Lignin (%)**
Hardwood stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nutshells	25-30	25-30	30-40
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat straw	30	50	15
Rice straw	32.1	24	18
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seeds hair	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste paper from chemicals Pulp	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Fresh bagasse	33.4	30	18.9
Swine waste	6	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

NA= Not Available

In degradation of lignocellulose, weak acids tend to remove lignin but result in poor hydrolysis of cellulose whereas strong acids occurs under relatively extreme corrosive conditions of high temperature and pH in which necessitate the use of expensive equipment (Howard *et al.,* 2003). The exact mechanism by which lignocellulose is degraded enzymatically is still not fully understood but significant advances have been made to gain insight into the microorganisms their lignocellulolytic genes and various enzymes involved in the process.

2.1.1 Lignin

Lignin can be defined as a hard material embedded in the cellulose matrix of vascular plant cell walls that functions as an important adaptation for support in terrestrial species (Campbell, 2002). It is found mostly between cells and also within the cells. Lignin holds cellulose and fibers together. The high concentration of this recalcitrant polymer is found in the middle lamella, where it acts as a cement between wood fibers, but it is also present in the layers of the call wall (especially the secondary cell wall), forming, together with hemicelluloses, an amorphous matrix in which the cellulose fibrils are embedded and protected against biodegradation. Its function is to regulate the transport of liquid in the living plant partly by reinforcing cell walls and keeping them from collapsing, partly by regulating the flow of liquid and it enables trees to grow taller and compete for sunshine (McCrady, 1991).

As in nature, lignin has been grouped into several types of characteristics such as, hardwoods, softwoods and grasses. Each type represents a lot of variations. Lignin differs from one species to another and also differs from one tissue to the next even in the same plant. Lignin is the major component of the middle lamella region of wood, but the most of the lignin is found within the secondary wall.

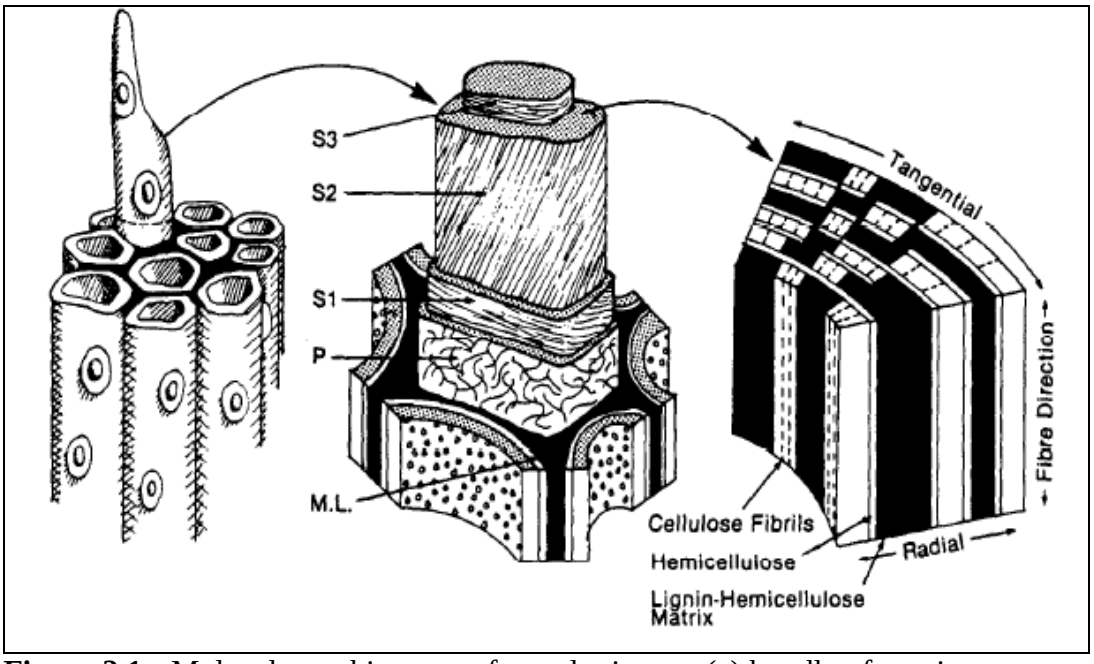

Figure 2.1: Molecular architecture of woody tissues: (a) bundle of contiguous wood cells, (b) wall layers in cut-away view of single cell, and (c) section of secondary wall illustrating the relationship of hemicellulose and lignin to the cellulose fibrils. Cell wall layers are P (primary): and S1, S2 and S3 (secondary). The middle lamella (M.L.) separates the cells (Kirk *et al.,* 1985).

In paper and pulp industries, lignin is the colored material that must be removed in pulp bleaching because it is what makes the mechanical pulp fibers stiff and makes newsprint turn yellow (Kirk *et al.,* 1985). Lignin is what makes the cellulose and hemicellulose in wood indigestible. Lignin is indigestible by mammalian and other animal enzymes but some fungi and bacteria are able to biodegrade the polymer (Wikepedia.org). Lignin is very resistant to the degradation because it being held together with the strong chemical bonds. Lignin is a complex polymer in which the building blocks are phenolic compounds. It contains three different aromatic alcohol units which are *coniferyl alcohol, p-courmaryl alcohol* and *sinapyl* alcohol (Howard *et al.,* 2003). Lignin macromolecules in which are mostly made of phenylpropanoid units linked each other through different kinds of bonds.

All lignin contain small amounts of incomplete or modified monolignols and other monomers are prominent in non-woody plants (Ralph *et al.*, 2001). The complexity of lignin is the main cause, why lignin is very hard to be degraded. The variety of units in lignin cannot be degraded by a single enzyme because different enzymes are needed to break each bond. The structure of lignin was finally understood only in 1960s, through pioneering work done primarily in Germany and Sweden.

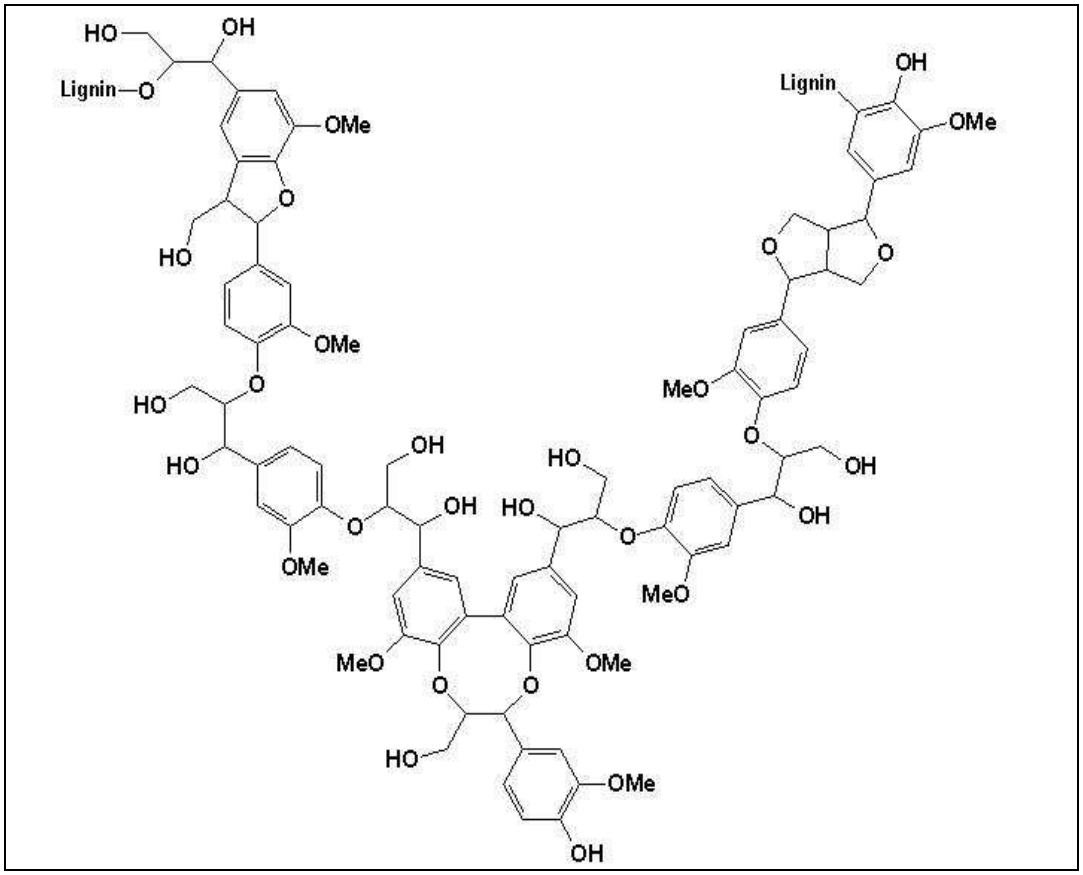

Figure 2.2: The structure of Lignin (Wikepedia.org).

2.2 Lignin Degrading Enzymes

Lignin has been found to have a very complex structure which contributes to the needed of these substrates to be degraded by more than one enzyme. Different

enzyme may be degrading lignin by cleaving different parts of the lignin structure. Tables 2.2 show the different types of enzyme which helps degrading lignin in different parts of its structure.

Table 2.2: Enzymes involved in the degradation of lignin (Hatakka, 1994)

Enzyme Activity, Abbreviation	Cofactor or Substrate, "Mediator"	Main Effect or Reaction
Lignin peroxidase, Lip	H_2O_2, veratry alcohol	Aromatic ring oxidized to cation radical
Manganese peroxidase, Mnp	H_2O_2, Mn, organic acid as chelator, thiols, unsaturated lipids	Mn(II) oxidized to Mn(III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Laccase, Lacc	O_2; mediators, e.g., hydroxybenzatriazole or ABTS	Phenol are oxidized to phenoxyl radicals; otherreactions in the presence of mediators.
Glyoxal oxidase, GLOX	glyoxal, methyl gloxal	Glyoxal oxidized to glyoxylic acid; H_2O_2 production
Aryl alcohol oxidase, AAO	Aromatic alcohols (anisyl, veratryl alcohol)	Aromatic alcohol oxidized to aldehydes; H_2O_2 production
Other H_2O_2 producing	Many organic compounds	O_2 reduced to H_2O_2

2.2.1 Lignin Peroxidase (Lip)

Lignin peroxidase is one of the enzymes which can degrade lignin. Lignin peroxidase is also known as ligninase. They are heme proteins, exhibiting Soret bonds in the visible range of 408 nm. This heme protein can easily been inhibited by the known heme inhibitor such as potassium cyanide and sodium azide (Howard *et al.,* 2003). It degrades lignin by oxidation activity and this oxidative activity has been found occurred in pH range of 4.0 – 8.0. Acidic pH condition is the optimal for lignin mineralization. A lignin peroxidase enzyme having a molecular weight of about 18 kD.

Enzyme activity that produces Cα-oxidation and Cα-Cβ cleavage of lignin and lignin degradation products, have a specific activity greater than 0.30 enzymes U/mg. (Crawford *et al.,* 1993). This enzyme is extracellular, oxidative, inducible by lignin, larch wood xylan, or related substrates and capable of attacking certain lignin substructure chemical bonds that are not degradable by known fungal lignin peroxidases. This bacterial enzyme has been proven that it works more effectively compared to the lignin peroxidase produced by fungi. This is due to the capability of bacterial enzyme of attacking certain lignin substructure chemical bonds that fungal lignin peroxidase cannot. Associated with primary growth of bacteria, it results the primary metabolic activity and not depends on other factors such as stress to induce production. The high contents on organic and inorganic nitrogen in culture media do not inhibit lignin degradation by the enzyme. The first enzyme reported in bacterial is *Phanerochaete chrysosporium* (Crawford *et al.,* 1993).

The mechanism of action of lignin peroxidase is just the same as other mechanism of peroxidases enzymes. The reaction is single electron oxidation of aromatic nucleito produce unstable species called radicals. These radicals undergo a variety of further reaction, many of which do not involve the enzyme (Kirk *et al.,* 1985).

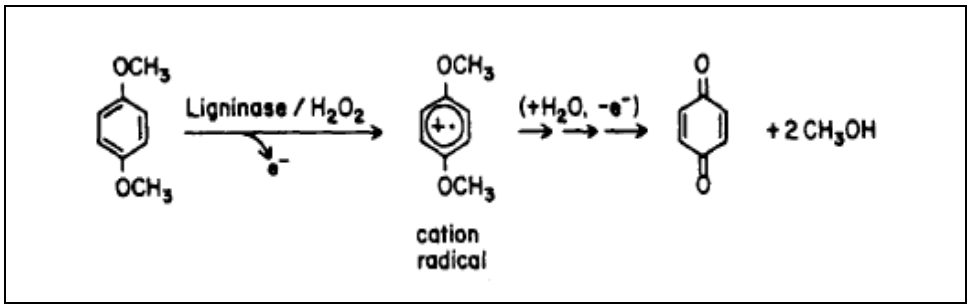

Figure 2.3: The mechanism of Ligninase (Kirk *et al.*, 1985)

2.2.2 Manganese Peroxidase (Mnp)

Besides lignin peroxidase, manganese peroxidase is also found as one of the lignin degrading enzymes. Manganese peroxidase is a heme containing enzyme which was first isolated from the extracellular medium of lignolytic cultures of white rot fungus *Phaenerochaete chrysosporium*, and it is considered to be a key enzyme in lignolysis by white rot fungi. It has been one of the important enzymes in delignification of kraft pulps (Gakkaishi, 1994). It increases the brightness of hardwood kraft pulp. The usage of manganese treatment anyway lowered the kappa number of kraft pulp. The mechanism of manganese peroxidase (Mnp) is they release the methanol from methoxyl groups on rings with free phenolic hydroxyls (Reid *et al*., 1998). However, manganese peroxidase partially oxidizes the lignin in the pulp but does not degrade it to soluble fragments. This cause maybe the reason of probably manganese peroxidase only function at an early stage in the degradation lignin was made. Manganese peroxidase requires H_2O_2 as a cosubstrate and catalyzes the oxidation of Mn^{2+} to Mn^{3+}. Mn^{3+} complexed with an organic acid acts as a primary agent in lignolysis. Manganese peroxides could degrade residual lignin in kraft pulp.

2.2.3 Laccase

Laccase have been known for many years in plant and fungi where they play variety of roles includingsynthesis of pigments, fruity-body morphogenesis and detoxification. Laccase was first isolated from the Japanese lacquer tree (*Rhus venicifera*) in 1883 (Chem.ox.ac.uk). Recently, laccase was found in bacteria. They are implicated in the degradation of lignin. The phenolic substrates provide electrons for the concomitant four electron reduction of atmospheric O_2 to water. This situation enables them to catalyze the one electron oxidation of a range of substrates. Currently the catalytic properties of laccases are being exploited for a range of technological applications such as the bioremediation of soils and water and the development of environmentally friendly processes in the pulp and paper industry (Chem.ox.ac.uk). However, laccase alone has a limit effect on lignin degradation.

It has been reported that the inclusion of a mediator such as ABTS (2,2'-azonobis(3-ethylbenzthiazoline-6-sulfonate) (Fernaud *et al.,* 2006), HBT(1-hydroxybenzotriazole), VLA(violuric acid), and TEMPO(2,2',6,6'-tetramethyl-piperidine-*N*-oxyl) can extend the substrate range of laccase to non-phenolic subunits of lignin (Fabbrini *et al.,* 2002). The degradation by laccase was accomplished when surfactants were added to the system. The type of laccase, redox mediator and surfactant affected the extent of degradation (Elegir *et al.,* 2005).

2.3 Lignin Degraders Pathway

Figure 2.4 shows the pathway of the lignin degarders. Laccase or ligninolytic peroxidases (LiP and MnP) produced by white rot fungi oxidize the lignin polymer, thereby generating aromatic radicals (a), These evolve in different non-enzymatic reactions, including C4-ether breakdown (b), aromatic ring cleavage (c), Cα-Cβ breakdown (d), and demethoxylation (e). The aromatic aldehydes released from Cα-

Cβ breakdown of lignin, or synthesized de novo by fungi (f, g) are the substrate for H_2O_2 generation by AAO in cyclic redox reactions involving also AAD. Phenoxy radicals from C4-ether breakdown (b) can repolymerize on the lignin polymer (h) if they are not first reduced by oxidases to phenolic compounds (i), as reported for AAO. The phenolic compounds formed can be again reoxidized by laccases or peroxidases (j). Phenoxy radicals can also be subjected to Cα-Cβ breakdown (k), yielding p-quinones. Quinones from g and/or k contribute to oxygen activation in redox cycling reactions involving QR, laccases, and peroxidases (l, m). This results in reduction of the ferric iron present in wood (n), either by superoxide cation radical or directly by the semiquinone radicals, and its reoxidation with concomitant reduction of H_2O_2 to hydroxyl free radical (OH·) (o). The latter is a very strong oxidizer that can initiate the attack on lignin (p) in the initial stages of wood decay, when the small size of pores in the still-intact cell wall prevents the penetration of ligninolytic enzymes. Then, lignin degradation proceeds by oxidative attack of the enzymes described above. In the final steps, simple products from lignin degradation enter the fungal hyphae and are incorporated into intracellular catabolic routes.

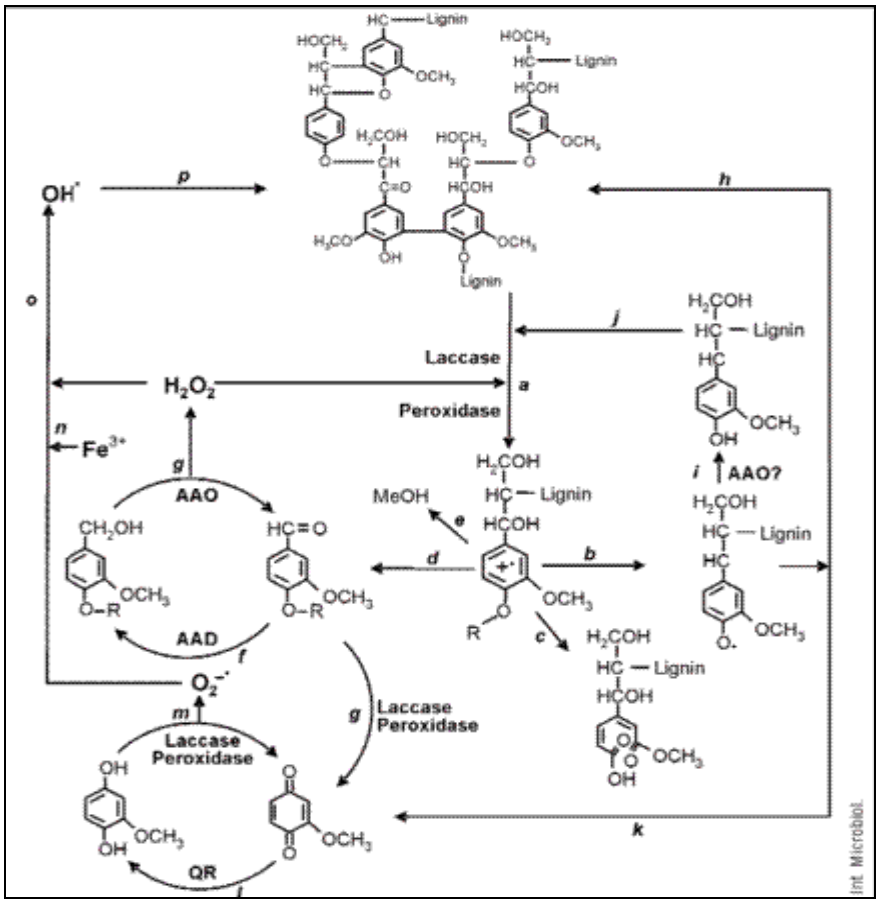

Figure 2.4: A scheme for lignin biodegradation including enzymatic reactions and oxygen activation.

2.4 Bacterial Lignin Degrader

Bacteria in soils appear in small sizes in which enable them to disperse readily with dust particles. Some of these bacteria can assimilate nitrogen from air (nitrogen fixation). Besides many bacteria tolerate or actually required the absence of oxygen (anoxia). Fertile topsoil might contain 10^9 living bacteria per gram of soil (Singer *et al.*, 2006). These bacteria cells might be divided every few hours under favorable conditions.

2.4.1 *Streptomyces sp.*

Streptomyces is the largest genus of Actinobacteria. Actinobacteria or Actinomycetes can be defined as a major group of filamentous bacteria, some of which are abundant in soils; once classified as intermediate between fungi and bacteria (Singer *et al.,* 2006). They are a group of gram-positive bacteria (*Wikipedia.org*). There are 3 genera (genus) under Actinomycetes which are A*ctinomyces*, *Nocardia* and *Streptomyces*. These organisms are the higher bacteria. *Actinomyces* are anaerobic while *Nocardia* and *Streptomyces* are aerobics (*pathmicro.med.sc.edu*). Streptomyces can be found predominantly in soil and in decaying vegetation and most produce spores (*Buchanan and Gibbons,* 1974). One of the lignin degradation enzymes can be found in these species. Streptomyces species that can produce lignin peroxidase are *Streptomyces viridosporus* and *Streptomyces badius*.

2.4.1.1 *Streptomyces viridosporus*

Streptomyces viridosporus is one of the species under the Actinobacteria group. It produced lignin peroxidase which degrades lignin. The degradation of hardwoods, softwoods and grasses lignin by *Streptomyces viridosporus* has been identified (*Crawford*, 1981). In some studies, *Streptomyces viridosporus* is grown on the lignocellulose and it has been found that both lignin and carbohydrate is degraded (*Barder et al.,* 1984).

2.4.1.2 *Streptomyces badius*

Same to the *Streptomyces viridosporus*, this bacterium also produced lignin peroxidase. Compare to the *Streptomyces viridosporus*, *Streptomyces badius* was less

lignin-like and increased substantially in average molecular weight over time (*Pubmedcentral.nih.gov*)

2.4.2 *Bacillus subtilis*

Bacillus subtilis is also one of the soil bacteria. It is a Gram-positive bacterium (*Wikipedia.org*). One of the characteristics shows by *Bacillus subtilis* is that this bacterium has a natural fungicidial activity and is employed as a biological control agent. Besides, it can convert explosives into harmless compounds of nitrogen, carbon dioxide, and water. It has been discovered that this species produced polyphenol oxidase enzyme. Polyphenol oxidase enzyme is also known as laccase. This enzyme degrades lignin as well as *Streptomyces sp.* (Korsten and Cook, 1996).

2.4.3 *Pseudomonas sp.*

Pseudomonas is a genus of gamma proteobacteria. It belongs to the family of pseudomonadaceae (Wikipedia.org). The characteristics of the pseudomonas species are, they are rod shaped, classified as Gram-negative bacteria, and also an aerobic bacteria. Enrichment technique yielded a lignin a degrading bacterium characterized as Pseudomonas sp. This organism was able to degrade acid, dioxane and fibre lignins which are the true representatives of native lignin. The direct polyphenol oxidase and laccase enzyme assays and the indirect ligninase assay with α-keto-γ-methyl thiol butyric acid and the concomitant release of phenols and sugars proved the organism's ability to degrade lignin (Uma *et al.,* 1979).

2.5 Fungal Lignin Degraders

2.5.1 *Phanerochaete chrysosporium*

Some features that might make it very useful have been discovered in *Phanerochaete chrysosporium*. The optimum temperature of this fungus is about 40 °C which means it can grow on wood chips in compost piles, which attain a very high temperature. This fungus produced lignin peroxidase and manganese peroxidase as well.

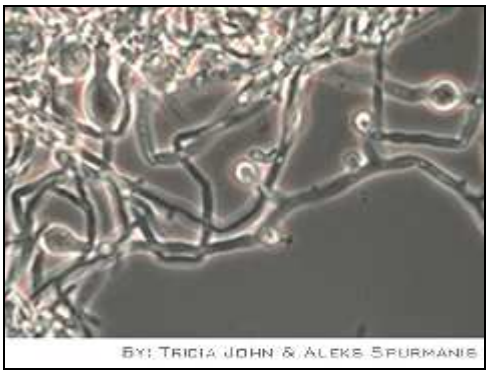

Figure 2.5: Microscopic view of *Phanerochaete chrysosporium*

Figure 2.6: The white rot fungi

2.6 Usage of Lignin Degrader

2.6.1 Biopulping and Biobleaching Industry

Paper pulp may be produced from a wide range of fibrous and woody (lignocellulosic) plant materials. Many of paper industries are still using the conventional methods. This is because of the high cost of the usage of microorganisms. But still many industries have used this approach in order to increase the quality of the products and also it is friendly to the environment. Microorganisms which produce enzymes are being used in biopulping and biobleaching industries in order to reduce the color due to residual lignin (Biomatnet.org).

Microbes were screened in order to identify the most appropriate microbial species for lignin degradation and decolorisation. The enzymatic system were searched for the production of peroxidase, lignin molecule attach (Lignin peroxidase, LiP, Manganese peroxidase, MnP, and laccase). LiP and MnP were produced on air-lift tyoe reactors. Those two enzymes were extracted from *Phanerochaete chrysoporium.* The usage of single enzymatic systems was not sufficient for pulping because it is hard to degrade lignin. The complex structures of lignins make them really hard to digest and they need to be treated by more than one enzyme (Shukla *et al.*,2004).

2.6.2 Bioremediation

Compare to the usage of chemicals in bioremediation process, the usage of fungi which have the ability to transform a wide variety of hazardous chemicals is safer to the human and also environment friendly. In degradation of lignin, the white rot fungi are unique. This is because they evolved nonspecific methods for the

degradation of lignin. Lignin degradation is, therefore, essentially a secondary metabolic process, not required for the main growth process (Lamar *et al.*, 1993) compared to abilities of lignin degradation fungi such as *Phanerochaete chrysosporium* to degrade PCP (Pentachlorophenyl) and creosote in soil.

Phanerochaete chrysosporium has been shown to effect the bioleaching of organic dyes (Nigam *et al.*, 1995). The role of lignin peroxidase and manganese peroxidase produced by *Phanerochaete chrysosporium* has been demonstrated in decolorization of olive mill waste water (Sami and Radhaune, 1995). *Phanerochaete chrysosporium* and microbial consortia were effective in color removal from textile dye effluents the fungus caused 80% decolorization in broth containing 2.5% of effluent. There was reduction in BOD and COD values.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Chemicals

All the chemicals used in this study were of analytical grade and all the chemicals were obtained from Fluka (Aldrich Chemical).

3.2 Collecting Sample Soil

3.2.1 Sources of Lignin Degrader Enzymes

Three **s**amples were taken from the soils around Universiti Malaysia Pahang (UMP). The test soil was fine sand collected from the depth of 30 cm from the ground surface. The sample soil is being dried and kept from contamination for 48 hours at 33°C.

3.3 Preparation of Cultivation Medium

3.3.1 Preparation of Agar Medium

A total of 0.2% (w/v) of nutrient agar was used. An amount of 28g agar powder was weigh and dissolved into 1000ml of ultrapure water and being heated on the heating plate. The schott bottle contained agar solution was covered with aluminium foil and prior to sterilization. Sterilization was carried out at 121°C for 20 minutes. After sterilization was completed the bottles was removed and it is allowed to cool enough so that the agar will begin to solidify. After that, the agar was poured into Petri dishes until the agar just covered the bottom of the Petri dish (approximately 20 ml). The Petri dish cover was placed on the Petri dish immediately and the agar was allowed to solidify (fully gel). After the plate of agar has solidified the plate is turned upside down and being leaved for 48 hours at room temperature to dry.

3.3.2 Preparation of Selective Agar

The selective agar comprises of lignin as the main component and minerals to support the growth of the lignin degrader microorganism. The selective agar medium consisted of 0.80 g/l lignin, 2g/l potassium nitrate (KNO_3), 1 g/l dipotassium hydrogen phosphate (K_2HPO_4), 0.5 g/l magnesium sulphate ($MgSO_4$), 3 g/l calsium carbonate ($CaCO_3$), 0.01 g/l ferum sulphate ($FeSO_4$) and 20 g of agar powder. The schott bottle was covered with aluminium foil and prior to sterilization. Sterilization was carried out at 121°C for 20 minutes. After sterilization was completed the bottles was removed and it was allowed to cool enough so that the agar will begin to solidify. After that, the agar was poured into Petri dishes until the agar just covered the bottom of the Petri dish (approximately 20 ml). The Petri dish cover was placed on the Petri dish immediately and the agar was allowed to solidify (fully gel). After the plate of agar had solidified, the plate was kept in 4°C before further use.

3.3.3 Preparation of Seed Culture Medium

In preparation of seed culture medium, the main components are lignin and minerals to support the growth of the lignin degrader microbes. The medium consists of 0.80 g/l lignin, 2g/l potassium nitrate (KNO_3), 1 g/l dipotassium hydrogen phosphate (K_2HPO_4), 0.5 g/l magnesium sulphate ($MgSO_4$), 3 g/l calsium carbonate ($CaCO_3$), and 0.01 g/l ferum sulphate ($FeSO_4$).

3.4 Preparation of Inoculum

A loopful of microbe from the agar medium was transferred into the 250 ml shake flask containing 30 ml of seed culture medium. The culture was incubated at 33°C for 36 hours, at 200 rpm to obtain the biomass cell. The bacterial cell was centrifuge at 5000 rpm, for 5 minutes. The cell pallets were washed twice with 0.85% (w/v) NaCl, and dissolved again in same solution. The mixture was homogenized and the optical density was read at 575 nm using spectrometer until it reaches 0.5. After that, 5ml of the solution is then added to 25 ml of seed culture in 250 ml shake flask and agin being incubated at 33°C for 36 hours, at 200 rpm.

3.5 Preparation of Crude Enzyme

5 ml of pellet dissolved in NaCl with OD of 0.5 is added with 25 ml of seed culture medium in 250 ml shake flask. The culture was incubated for 24 hours at 33°C, and 200 rpm. The sample was centrifuged at 5000 rpm for 5 minutes. The clear supernatant was used as crude enzyme. This crude enzyme was used for the characterization of enzyme which is the effect of pH and the effect of temperature towards the efficiency of the enzyme.

3.6 Screening

3.6.1 Sample Dilution and Primary Screening

1 gram of soil sample was weighed out and being placed in tube 1 containing of 9 ml of saline solution. The suspension was then being thoroughly vortexes for about 1 minute. A 10-fold serial dilution of the soil was made by transferring 1 ml of solution from each tube to a new tube to achieve a final dilution of 1: 1000 000 in tube 6. From each tube, 1 ml of suspension was pipette and poured into agar plate prepared in the early stage. An alcohol-flamed hockey stick was used to spread the suspension poured in the Petri dish. The plates were then incubated at 30°C for 5-7 days.

3.6.2 Second and Third Screening

From the first screened plate, five colonies being selected and each was streaked for isolation onto selective agar plate. The plates were then being incubated at 30°C for 5-7 days. The colony was transferred weekly for 10 times to ensure that the culture was pure enough for morphology and physiological test. Figure below shows the streaking method. For the streaking method, the loop was flame sterilized and then a loopful of colony was removed from nutrient agar plate. The streak was made over a sterile agar plate to spread out the organism. By following the initial streak, subsequent streak was made at angles to it.

Figure 3.1: Streaking plate method

3.7 Preservation of Microbes

A 10% (w/v) solution of glycerol is prepared and 2 ml of it is dispensed into 4 ml capacity Wheaton borosilicate glass vials fitted with rubber lined caps. The vials were autoclaved twice for 45 minutes each. Transfer tubes were autoclaved for 25 minutes. Several plugs were cut and deposited into each of the vials, which were then frozen and stored at -85°C.

3.8 Characterization of Microorganisms

3.8.1 Morphological Test

3.8.1.1 Simple Staining

The slide was cleaned using suitable cleaning material. The slide was gently dried with a lint free cloth. A very small drop of distilled water was placed on the surface of the slide. Then, aseptically, a small amount of the culture was removed from the agar surface and just touches it several times to the drop of water until it just turns cloudy. The remaining bacteria were burnt off of the loop. By using a loop, the suspension was spread over the entire slide to form a thin film. This thin suspension was allowed to completely air dry. The slide was passed through the flame of the Bunsen burner for 3 or 4 times to heat-fix. After that, the smear was then covered with methylene blue. The dye remain on the smear was allowed for approximately 1 minute. The excess stain was washed off of the slide using bibulous paper. The slide was picked up by one end and being hold at an angle over the staining tray. By using the distilled water wash bottle, the excess methylene blue from the slide was gently washed by directing a gentle stream of water over the surface of the slide. Any stain that got on the bottom of the slide was washed off as well. The excess stain was blotted off using bibulous paper. Be sure that the slide was not being rubbed rather the slide was placed between two sheets of bibulous paper and was pressed down

gently. The slide was then examined under the brightfield microscope. The shape, arrangement and approximate size of the microorganisms were recorded.

3.8.1.2 Gram Staining

The smear was prepared. The slide was heated for a few seconds until it becomes hot to the touch so that bacteria were firmly mounted to the slide. The primary stain which was crystal violet (2g of 90% crystal violet dissolved in 20 ml of 95% ethyl alcohol) was added and incubated for 1 minute. The Gram iodine (1 g of iodine, 2 g of potassium iodide, dissolved in 300 ml of distilled water) was added for 1 minute. Then it was washed with ethanol and acetone, the decolorizer (50% ethyl alcohol, 50% acetone). The Gram-positive bacteria were retained as the primary stain while the Gram-negative bacteria were lost the primary stain and appeared colorless. The secondary stain which was safranin was added and was incubated for 1 minute and then being washed with water for a maximum of 5 seconds. The shape, arrangement and approximate size of the microorganisms were recorded.

3.8.1.3 Spore Staining

A boiling water bath was prepared. A bacterial smear and heat fix was prepared. The slide was placed on the staining rack over the boiling water. The slide was flooded with Malachite green and was then being steamed for 3 minutes by adding the additional stain as needed to prevent drying. The slide was removed and was allowed to cool. The excess stain was removed by washing it with water. The slide was then flooded with safranin for 2 minutes and then was washed with water. The slide was blotted dry with bibulous paper. The shape, arrangement and approximate size of microorganisms were recorded.

3.9 Characterization of enzymes

3.9.1 Effect of pH

Crude enzyme was harvested through the enzyme assay and was tested for the effect of the pH. A range of pH, 6 to 9 was used to test the stability of enzyme. Buffer solution was prepared by using K_2HPO_4 and KH_2PO_4. 0.2 ml crude enzymes were added to 1.8 ml of substrate which contain lignin and buffer solution. Four different pH was incubated at constant Temperature for about 15 minutes. The enzyme efficiency under this pH was tested by using DNS method. Then, the enzyme activity was calculated.

$$Enzyme\ activity = \frac{\mu mol\ of\ sugar}{2.0\ ml \times 15\ min}$$

3.9.2 Effect of Temperature

The temperature range examined was, 40°C, 50°C, 60°C, 65°C and 70 °C. Buffer solution of the optimum pH was added with lignin substrate which then was added with 0.2 ml of crude enzyme. The solution was then incubated at those different temperatures for about 15 minutes. After the incubation was done for 15 minutes for each temperature, the solution was then added with 3 ml of DNS reagents and was boiled for about 5 minutes. The optical density was read at wavelength of 575 nm using spectrophotometer. The highest value of glucose obtained represents the best temperature of the enzyme. Then, the enzyme activity was calculated.

3.10 Analysis Procedure

3.10.1 DNS Method

In order to prepare dinitrosalicyclic acid (DNS) reagent, 100 ml of 5% (w/v) of 3, 5- dinitrosalicyclic acid is mixed in 2 M sodium hydroxide (NaOH) with 250 ml of 60% (w/v) sodium tartrate and made the total volume up to 500 ml with distilled water. 3ml of DNS was added to the solution which contained substrate and crude enzyme that was incubated for 15 minutes in the water bath at set temperature. After that, the solution was then being boiled for about 5 minutes before the optical density of the solution was read at 575 nm wavelength using spectrophotometer. In preparation of the standard curve of glucose, a different concentration of glucose was prepared which are, 2000 μg/ml, 1500 μg/ml, 1000 μg/ml, 500 μg/ml and 200 μg/ml. 2 ml of each concentration was placed into 5 different test tubes. After that, 3ml of DNS reagents was added to each of tube and was boiled for about 5 minutes. The optical density of the solution was then checked and the absorbance of the solution was determined.

3.10.2 Protein Assay

3.10.2.1 Lowry Protein Assay Method

Lowry reagents was prepared by mixing of one volume reagent B (which contains 0.5% cooper sulphate pentahydrate, 1% sodium potassium tartrate) with 50 volumes of reagent A (which contains 2% sodium carbonate, 0.4% NaOH). This was assuming to be reagent 1. Then reagent 2 was Folin-Ciocalteu. Folin-Ciocalteu was diluted with an equal volume of water. In order to quantify the protein, 0.2 ml of crude enzyme was placed into a test tube. After that, 1ml of Lowry reagent was added to the crude enzyme and was left for 10 minutes. Folin reagent was added to the solution and was incubated for 30 minutes at room temperature. After incubation

completed, the optical density of the solution was read at wavelength of 575 nm using spectrophotometer and the determination of protein contents in the crude enzyme accomplished. The results were then being recorded. For the preparation of standard curve of the Lowry Protein assay, Bovine Serum Albumin (BSA) was used. A different concentration of BSA was prepared which are 2000 μg/ml, 1500 μg/ml, 1000 μg/ml, 500 μg/ml and 200 μg/ml. 2 ml of each concentration was placed into 5 different test tubes. Each test tube was added with Lowry reagent 1 and was incubated for 10 minutes before it was added with Folin reagent and was continued to be incubated at room temperature for about 30 minutes. After incubation was completed, the optical density of each solution in each tube was read at wavelength of 575 nm and the results were recorded.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 First Cultivation

After 4-5 days of incubation in 33°C environments, the growth can be seen on the surface of the nutrient agar. The plate of the first dilution was fully covered with the growth of microorganisms while there were no growths on the surface of the agar plate containing of fifth and sixth dilution. It shows that there were no microorganisms in the fifth and sixth dilution sample. Figure below shows one of the results obtained from the first cultivated media.

Figure 4.1: Agar medium was fully covered by the growth of microorganisms

Figure 4.2: The growth of microorganisms from the 4th dilution sample on the agar medium

From the Figure 4.2 it shows that, for the fourth diluted sample, the growth of the microorganism on the nutrient agar plate was clearly seen.

4.2 Primary and Secondary Screening

For the first screening, five colonies from each plate were transferred onto the new nutrient agar plate using streaking method. This process was held in the laminar flow in order to avoid the contamination. The transferred microorganisms were incubated for 5-7 days in 33°C incubator. Figure 4.3 below show the first screening of the microorganism from the first cultivated medium. It shows that those microorganisms started to growth in colonies. There are many colonies visible on the surface of the nutrient agar plate Screening is continuously performed until the single colony is formed on the nutrient agar.

Figure 4.3: First screening onto the nutrient agar

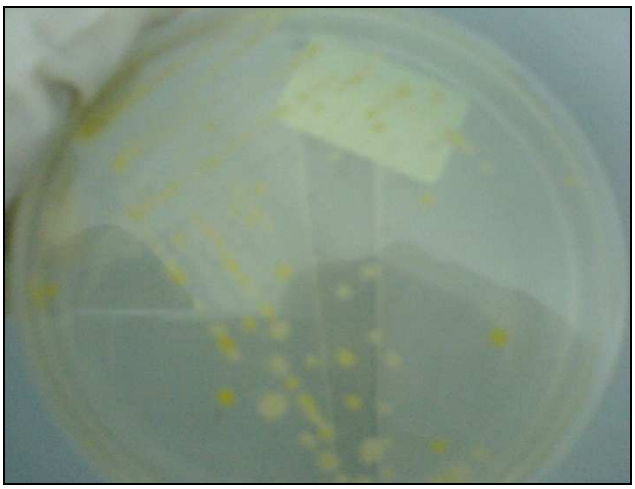

Figure 4.4: The microorganisms start to form single colony on the agar plate

4.3 Lignin Degrading Microbes

After the formation of single colony on the nutrient agar medium, those colonies were transferred onto the selective agar prepared. The selective agar contained lignin in order to let only the lignin degrader to grow and degrade lignin on the selective agar. The first transferred microorganism was incubated for 14 days before the first growth can bee seen on the surface of the selective agar. This was due to the lignin degraders itself which takes a long time for it to grow. After that, these microorganisms that grown on the selective agar were transferred weekly onto new

selective agar in order to keep the microorganisms in a condition that provides sufficient nutrients for them to grow and also in order to get the pure strain of the microorganisms found. Since the first time those microorganisms have been transferred onto the selective agar, it has been counted that this microorganisms have been going through the process for 7-8 times. Figure below shows the microorganisms obtained on the surface of the selective agar plate.

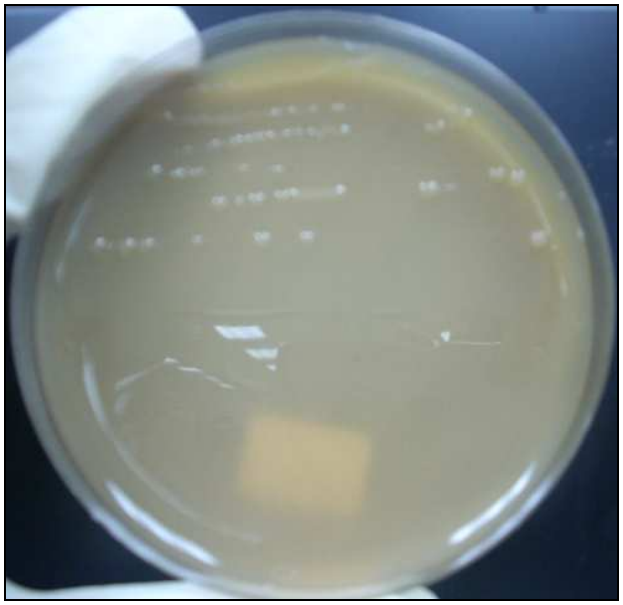

Figure 4.5: Single colonies formed on the surface of the selective agar

4.4 Characterization of Microorganisms

The single colony formed on the selective agar was then being tested with morphological test in order to identify and characterized the shapes, sizes, classification on the genus of the microorganisms and arrangements of the microorganisms obtained.

4.4.1 Morphological Tests

4.4.1.1 Simple Staining

Before staining can be performed, bacterial smear was prepared first. The purpose of the smear preparation is to place an appropriate concentration of cells on the slide and fixed them so that they do not wash off during the subsequent staining procedure. It is called simple staining because of the use of only one dye during staining. As for this experiment, methylene blue was used for simple staining. Simple staining reacted with all microbes in an identical fashion. They are useful for increasing the contrast of the bacterial cells from the background. Basic dye such as methylene blue, crystal violet, safranin and basic fuschin are positively charge which therefore reacts with material that is negatively charge. The surface of the bacteria at neutral pH is negatively charge. Figure 4.5 shows the stained bacteria under microscopic view with 100x magnification.

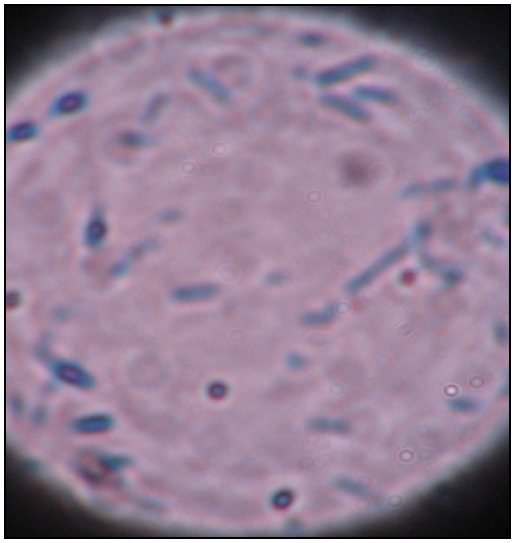

Figure 4.6: Simple Stained bacteria under microscopic view

The bacteria were dyed in blue color. The shape of the bacteria is long, narrow and same as the *Bacillus sp.* It presents as unicells bacteria. Also present was

microbes with the shape exactly the same shape of yeast which budding or branching hyphae form was seen.

4.4.1.2 Gram Staining

The theory of the gram staining was base on the cell wall construction. Gram positive and gram negative bacteria stain differently because of the structure of their cell walls. Gram positive bacteria and yeast stained in purple while gram negative bacteria and host cells stain pink. Figure 4.7 shows the result from the gram staining method. From Figure 4.7 it was found that there were mix populations in a colony which consisted of gram positive bacteria and yeast. The bacteria with long and narrow shape stained purple and the same colour goes with yeast. *Bacillus sp.* found to be gram positive bacteria. However, many bacteria such as *Bacillus sp.* rapidly become gram negative bacteria when entered the stationary phase of growth. (*Todar's online textbook of Bacteriology, retrieved on 18th April 2008*)

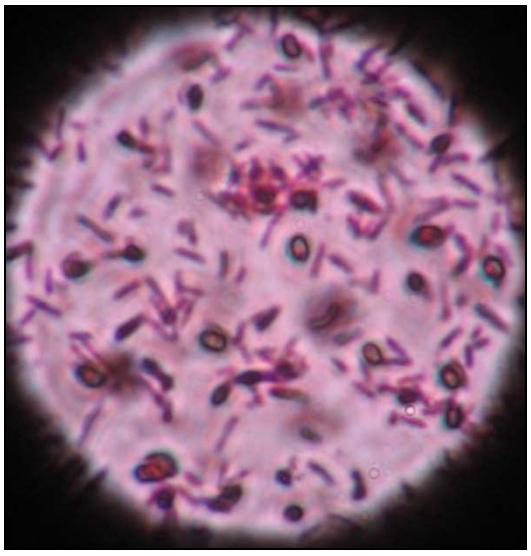

Figure 4.7: Gram stained bacteria under microscopic view

4.4.1.3 Spore Staining

From Figure 4.8 it can be seen and identified that in this field there were endospores forming bacteria and yeast. The yeast visible inside the field had been identified as a yield from sporulation process being through by *Bacillus sp.* It was found that, bacteria in this field were sporeformers bacteria. It strengthens the observation that the bacteria found is *Bacillus sp.* Spores and some of the *Baccilus sp* present in the picture were stained green while the vegetative cell (*Bacillus sp*. which not yet forming spores) were stained red. Cell or microorganisms that produced spore known as sporeformers posses remarkable resistance to heat, dryness, irradiation and many chemical agents. Each cell can only produced one endospore. Usually, cells of *Bacillus sp.* are the one which produced endospore. Endospores do not form normally during the active growth and cell division. Their differentiations usually begin when population of vegetative cells passes out of the exponential phase of growth. This situation occurred because of the nutrient depletion and in other to continuously survive, they used to form endospore. Only one endospore can be formed per vegetative cell.

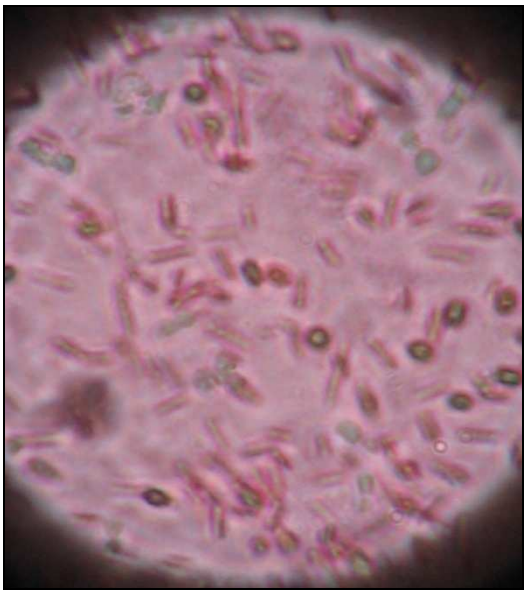

Figure 4.8: Spore stained bacteria under microscopic view

4.5 Characterization of Enzyme

4.5.1 The Effect of pH

The results obtained are shown in Table 4.1.

Table 4.1 Enzyme activity at different pH

pH	Optical Density (OD)	Enzyme Activity, U (µmol/ml/min)	Glucose Concentration (µg/ml)
6	1.102	405.00	1215
7	1.206	466.67	1400
8	0.998	366.67	1100
9	1.093	403.33	1210

From the data obtained in Table 4.1, the highest absorbance was found to be 1.209 which is obtained from buffer with pH 7. The value of absorbance shows the amount of simple sugar formed in the solution. The higher the absorbance, the higher the sugar contents in the solution. Also it shows that the higher the absorbance value obtained, the higher the enzyme activity. Enzymes contain in the solution have degraded lignin and converted it into simple sugars. Those absorbance is then was used to indicate the glucose concentration in the solution. The optimum pH is found to be in range of pH 6 and pH 7 due to the environmental condition of the microorganism's original place. Also, according to the Brock, (2006) Biology of Microorganisms, eleventh edition, bacteria from soil usually growth in range of pH 5-8. The best pH obtained is then was continued for the analysis of the effect of temperature to enzyme activity. From the previous study, it was also found that the optimum pH for soil's bacteria was in range of 7.5 to 9.0. (Kristjansson, 1992)

4.5.2 The effect of Temperature

The results obtained are shown in Table 4.2.

Table 4.2: Enzyme activity at difference temperatures

pH	Temperature (°C)	Optical Density (OD)	Enzyme Activity, U (µmol/ml/min)	Glucose Concentration (µg/ml)
6	33	1.102	405.00	1215
	40	0.978	366.67	1100
	50	1.478	518.33	1555
	60	1.220	455.00	1365
	65	1.200	446.67	1340
	70	1.193	445.00	1335
7	33	1.206	448.33	1345
	40	1.286	478.33	1435
	50	1.570	588.33	1765
	60	1.048	390.00	1170
	65	1.010	376.67	1130
	70	0.923	345.00	1035

From the results show in Table 4.2, it recovered that at pH 6, the higher absorbance obtained is at temperature of 50°C. Same goes with pH 7. At temperature 50°C, the enzyme activity is high and yet so many simple sugars formed as a result of lignin degradation. Also, results were clearly pictured in the Figure 4.9 and Figure 4.10 below. From the results obtained, it can be concluded that, the optimum temperature for the enzyme activity is 50°C. It shows that, the bacteria found is a thermophilic bacterium which can survive in range of temperature of 40-85°C. Prokaryotic organisms in general are able to grow at temperatures higher than those at which eukaryotes can grow. Their enzymes are much more stable to heat than those of mesophiles. That is why they function optimally at this temperature. Thermophiles typically have lipids rich in unsaturated fatty acids, thus making the membranes to remain stable and function at high temperatures. (Brock, 2006). From the previous study it was found that the optimum temperature for lignin degrader was at 45°C. (Ruijssenaars *et al.*, 2004).

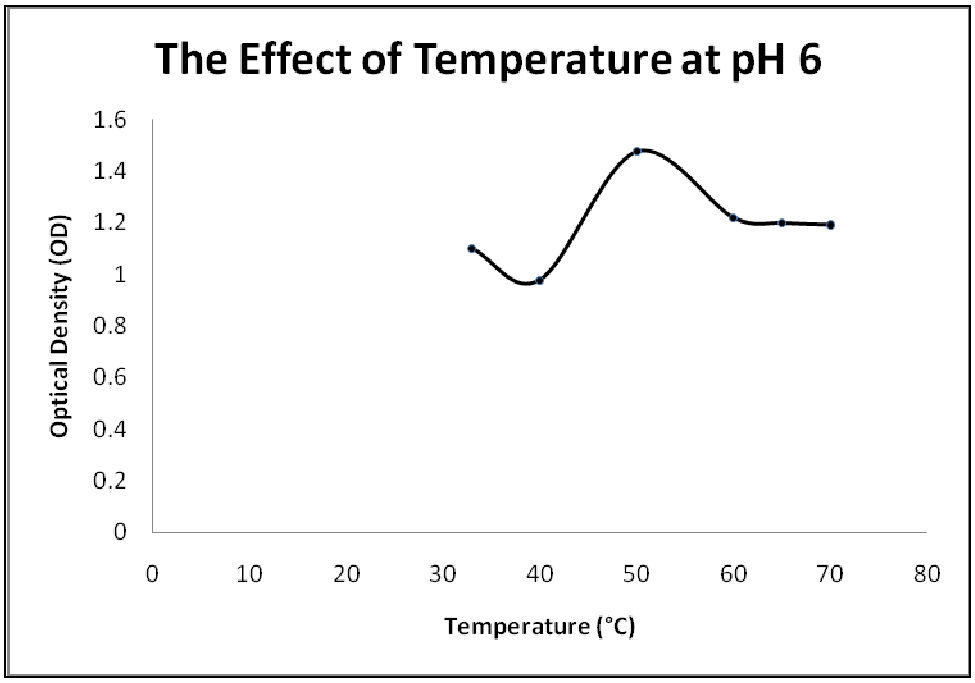

Figure 4.9: The Effect of Temperature at pH 6

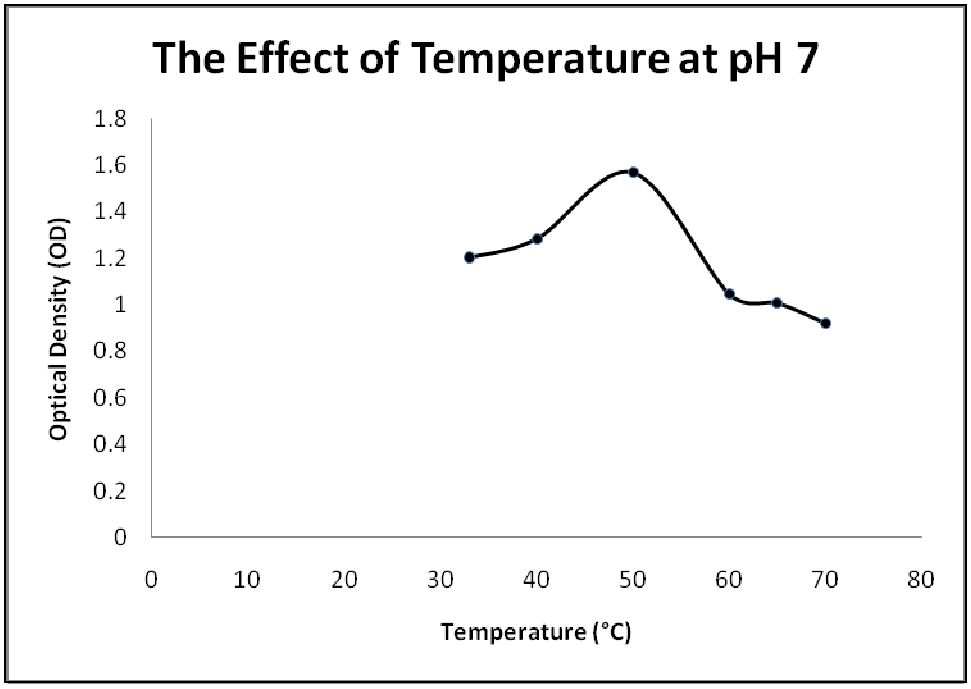

Figure 4.10: The Effect of Temperature at pH 7

4.6 Protein Contents

Table 4.3 shows the result for protein contents determined in the crude enzyme.

Table 4.3: Protein contents

Optical Density (OD)	**Protein Concentration (µg/ml)**
1.481	1590

From the result shown in the table 4.3, it was found that protein concentration in the crude enzyme was 1590 µg/ml with optical density was 1.481.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

Microorganisms from these samples were screened until single colonies were obtained. Colonies was tested with simple staining, gram staining, and spore staining. The shape that been found on the field was long and narrow shape which represent *Bacillus sp.* bacteria. Also found on the field was yeast which identified by the shape of budding and branching hyphae form.

From gram staining method, it can be concluded that the lignin degrader bacteria found on the field was a gram positive bacteria or more accurately the bacteria found was *Bacillus sp*. *Bacillus sp.* found was identified as gram positive bacteria since it was a form of long and narrow and the stain retained purple. Those spore forming bacteria was found in this field due to the long period of incubations which encourage the bacteria to formed spore in order to survive in the limited nutrient environment and also was became the heat and dryness resistant bacteria. Only microorganisms which produce spore will have those characteristics.

The optimum pH found based to the characterization of lignin degrading enzymes is pH 7. This is probable due to the commonly soil bacteria growth in pH neutral condition. The enzyme activity analyzed in the pH 7 was higher compare to the others. While the temperature optimum for the lignin degrading enzymes was

found maximum at temperature 50C for both pHs 6 and pH 7. Overall results showed that maximum ligninase activity was observed at pH 7 and 50C, which was at 588.3 μmol/ml/min.

5.2 Recommendation

Several recommendations for further studies are as follows:

a) Study on screening the lignin degrader, the selective can be divided into 3 types of selective agar which are 1) Peptone-glucose-acid-agar medium to screen bacteria, 2) Yeast extract agar selective, to screen fungi and 3) Actinomycete Isolation Agar which will screen actinomycetes.
b) Polymerase Chain Reaction (PCR) can be performed in order to recognize and classify the species of the bacteria being found.
c) In order to increase the production of enzyme, the bacteria must be incubated at the optimum pH and temperature.

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APPENDICES

Appendix A1

1. SAMPLE DILUTIONS

Preparation of saline solutions:

Apparatus
- 200 ml beaker
- Pipette
- Tips
- 18 test tubes
- Test tube racks
- Aluminium foil
- 500 ml Schott bottle
- Measuring cylinders

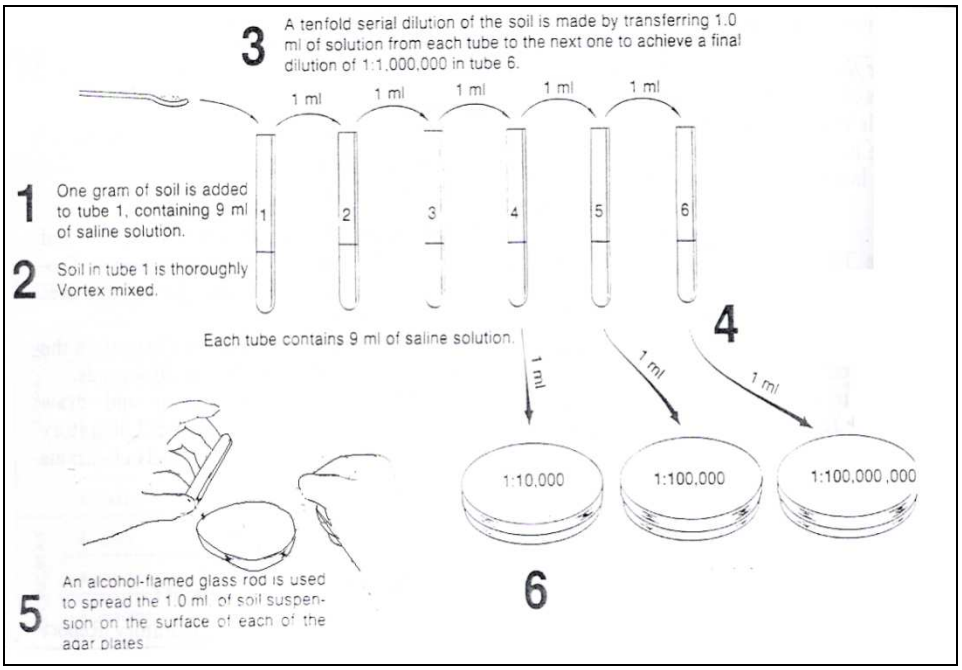
All the apparatus is sterilized at 121°C for 20 minutes

Chemicals/Solutions
- 0.85 g of NaCl
- 100 ml of ultrapure water

0.85 g of NaCl is dissolved in 100 ml of ultrapure water and well mixed.

Appendix A2

Methods for Sample Dilution and Primary Screening



2. SCREENING METHOD

Preparation of Nutrient Agar

Apparatus

- 1000 ml schott bottle
- Aluminum foil
- Heating plate
- Magnetic stirrer bar
- Spatula
- Analytical balance
- Weighing boat

Chemicals

- 28 gram nutrient agar powder
- 1000ml ultrapure water

28 gram of nutrient agar powder is dissolved in the 1000ml of ultrapure water while heating. The nutrient agar is well stirred and then is sterilized at 121°C for 20 minutes.

Appendix A3

Preparation of Selective Agar Medium

Apparatus

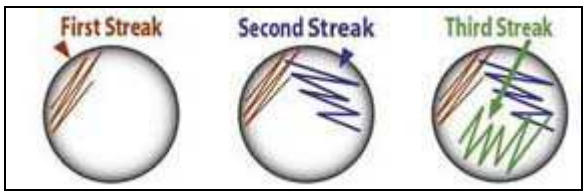
- 1000 ml schott bottle
- Aluminum foil
- Heating plate
- Magnetic stirrer bar
- Spatula
- Analytical balance
- Weighing boat

Chemicals

- 0.80 g/l lignin,
- 2g/l Potassium nitrate (KNO_3),
- 1 g/l Dipotassium Hydrogen Phosphate (K_2HPO_4),
- 0.5 g/l Magnesium Sulphate ($MgSO_4$),
- 3 g/l Calsium Carbonate ($CaCO_3$),
- 0.01 g/l Ferum Sulphate ($FeSO_4$) and
- 20 g of agar powder
- 1000 ml ultrapure water

1000 ml of ultrapure water is heated on the heating plate. While heating, the solution is stirred well using magnetic stirrer bar and all of the measured chemicals is added ang let it dissolved in the heated ultrapure water. After that, the selective agar medium is sterilized at 121°C for 20 minutes.

3. STREAKING METHOD



Colonies is transferred from one agar medium to another by streaking method showed in the picture above.

Appendix A4

4. MORPHOLOGY TESTS

Simple Staining Method

1) Slide is cleaned using lint cloth & gently dried
2) Small drop of distilled water is placed on the surface of the slide
3) Small amount of the culture is removed and touched the drop of water
4) Suspension is spread over the entire slide to form thin film
5) Smear is then covered with methylene blue and the dye is let to remain for 1 minute
6) Excess stained is washed off using filter paper
7) Slide is then examined under brightfiled microscope

Gram Staining Method

1) Smear is prepared
2) Crystal violet is added & incubated for 1 minute
3) Gram iodine is added for 1 minute
4) It is the washed with ethanol-acetone
5) Secondary stain, safranin is added & incubated for 1 minute and then being washed with water for a max of 5 seconds
6) Slide is then examined under brightfield microscope

Four reagents that is used for gram staining

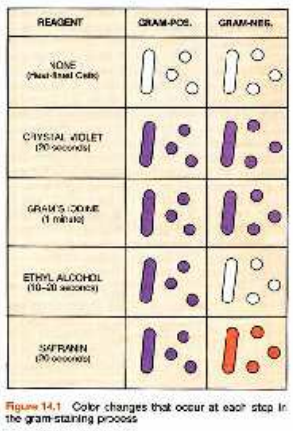


Figure 14.1 Color changes that occur at each step in the gram-staining process

Appendix A5

Spore Staining Method

1) boiling water bath is prepared
2) bacterial smear is prepared
3) the slide is flooded with Malachite green and steamed for 3 minutes
4) excess stain is washed using water
5) slide is examined under brightfield microscope

Appendix A6

5. CHARACTERIZATION O ENZYMES

Preparation of Seed Culture Medium

Apparatus

- 1000 ml schott bottle
- Aluminum foil
- Heating plate
- Magnetic stirrer bar
- Spatula
- Analytical balance
- Weighing boat

Chemicals

- 0.80 g/l lignin,
- 2g/l Potassium nitrate (KNO_3),
- 1 g/l Dipotassium Hydrogen Phosphate (K_2HPO_4),
- 0.5 g/l Magnesium Sulphate ($MgSO_4$),
- 3 g/l Calsium Carbonate ($CaCO_3$),
- 0.01 g/l Ferum Sulphate ($FeSO_4$) and
- 1000 ml ultrapure water

1000 ml of ultrapure water is heated on the heating plate. While heating, the solution is stirred well using magnetic stirrer bar and all of the measured chemicals is added ang let it dissolved in the heated ultrapure water. After that, the selective agar medium is sterilized at 121°C for 20 minutes.

Preparation of inoculums

1) A loopful of microbe from agar medium is transferred into 250 ml shake flask containing 30 ml of seed culture medium.
2) Culture was incubated for 36 hours at 33°C and 200 rpm to obtain biomass cell

Preparation of crude enzymes

1) 10 % of inoculum is transferred into 250 ml shake flask
2) The culture is incubated for 24 hrs at 33°C and 200 rpm
3) Sample is then centrifuge at 5000rpm for 5 minutes
4) The clear supernatant is used as the crude enzyme

Appendix A7

6. ENZYME ANALYSIS

Phosphate buffer

pH	Volume of 0.1M K_2HPO_4 (ml)	Volume of 0.1M KH_2PO_4 (ml)
6.0	13.2	86.8
7.0	61.5	38.5

Dilute the combined stock solution into 500 ml of distilled water.

Tris Buffer

pH	Volume of 2M HCl (ml)	Volume of Tris Solution (ml)
8.0	3.2	19.6
9.0	2	37

Dilute the combined stock solution into 500 ml of distilled water

DNS Method

1) 0.2 ml of crude enzyme is incubated with substrate for 15 minutes at 33°C (substrate contained of 0.5 % (w/v) alkaline lignin being dissolved into 1.8 ml of 0.05 M phosphate buffer with pH 7)
2) After that 3 ml of DNS reagent is added
3) The solution is being boiled for about 5 minutes
4) The optical density is checked using spectrophotometer at wavelength of 575 nm.

Appendix A8

Lowry Protein Method

Stock solution

Lowry A: 0.5% cooper sulphate pentahydrate, 1% sodium potassium tartrate
Lowry B: 2% sodium carbonate, 0.4% NaOH
Folin Ciocalteu: diluted with an equal volume of water

1) 2ml sample per tube (sample+buffer=2ml)
2) 1.0ml of lowry stock solution is added to each tube
3) Solutions is then incubated at room temperature for 10 min
4) 0.1 ml of Folin's reagent is added to each tube
5) The solution is the incubated at room temperature for 30 min
6) Read in spectrophotometer at 575 nm

Appendix B1

Standard Curve of DNS Method

The standard curve for glucose was being calibrated using the DNS method with different concentration of glucose. The calibrating data was being collected and the data are as the following:

Table B1: Results of absorbance based on glucose concentration

Glucose Concentration (µg/ml)	Optical Density (OD)
0	0.000
200	0.185
500	0.475
1000	0.839
1500	1.372
2000	1.802

The data collected is plotted into a graph, absorbance versus glucose concentration, where further analysis, the absorbance is checked and the concentration of glucose inside the sample can be determined. The plotted graph is as the following:

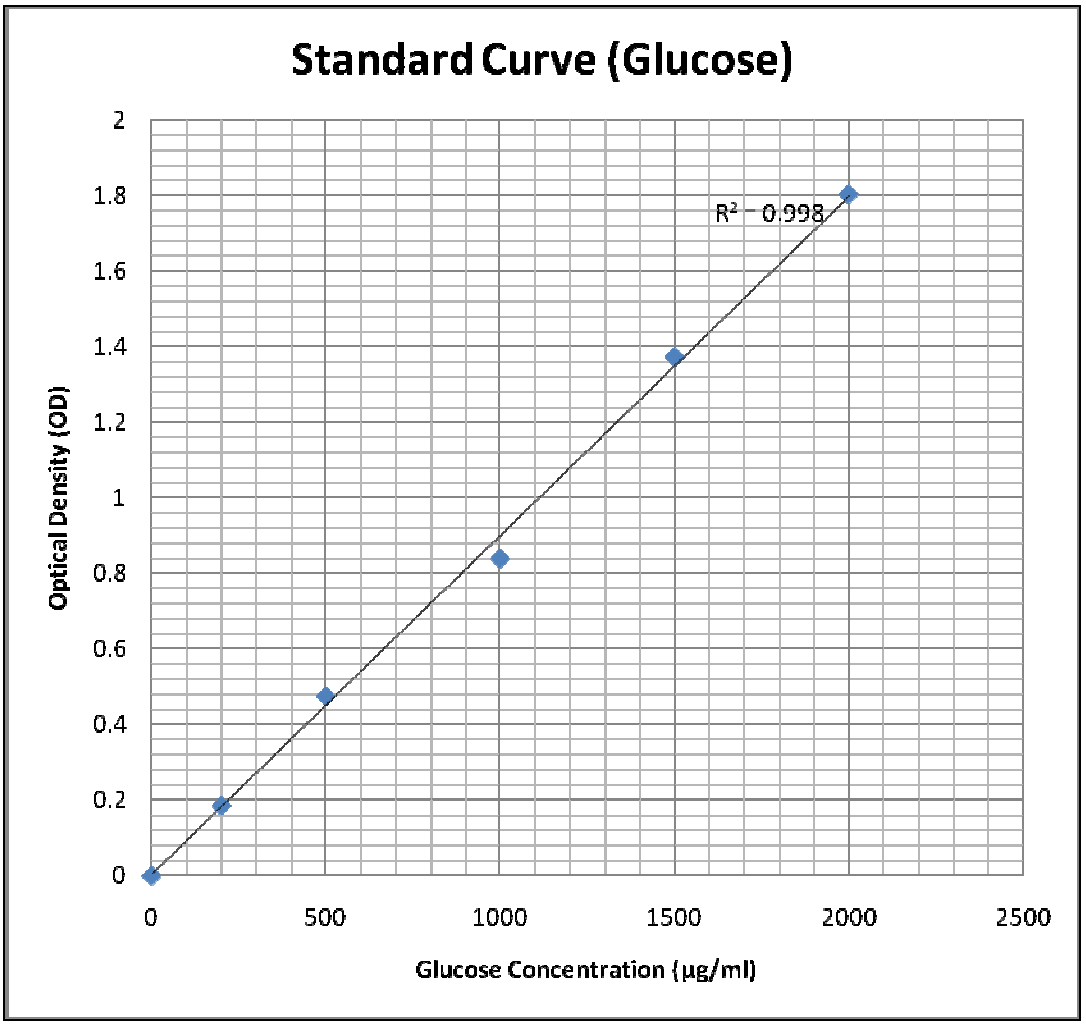


Figure B1: Standard Curve for Glucose

Appendix B2

Standard Curve of Lowry Method

Standard curve for Lowry method has been made for tests of protein concentration inside a solution. The concentration and absorbance was recorded in order to plot the curve. The following was the data collected to calibrate the curve:

Table B2: Results of absorbance based on protein concentration

BSA Concentration (μg/ml)	Optical Density (OD)
0	0
200	0.336
500	0.688
1000	1.079
1500	1.489
2000	1.659

The data collected is plotted into a graph, absorbance versus concentration, where further analysis, we will only check on the absorbance and we will know the concentration of protein inside the sample. The plotted graph is as the following:

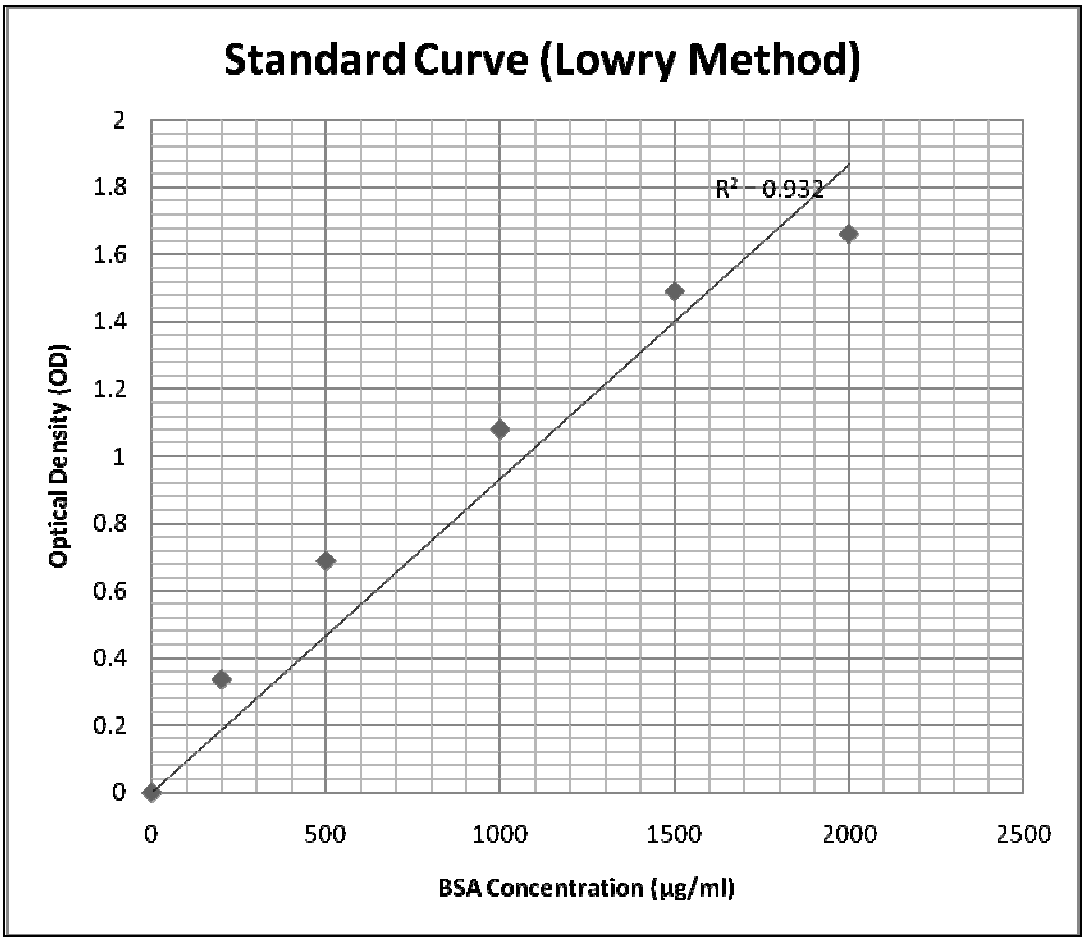


Figure B2: Standard curve for Lowry method