SCREENING OF LIGNIN DEGRADER FROM SOIL

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A thesis submitted in fulfilment of the
requirements for the award of the degree of
Bachelor of Chemical Engineering

FACULTY OF CHEMICAL ENGINEERING AND NATURAL RESOURCES
UNIVERSITY COLLEGE OF ENGINEERING AND TECHNOLOGY MALAYSIA

NOVEMBER 2006

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

Signature : .....

Name : Yeoh Teik Loon

Date : 20th November 2006

ACKNOWLEDGEMENTS

First of all, I would like to forward my appreciation to my father, mother and my sister for giving me endless support in every possible ways. Without them, I would never be able to be the person I am today.

This thesis is a result of one year of work, whereby many have contributed in helping me realize this thesis. I wish this project may contribute to science and technology.

I would like to thank my supervisor, Miss Wan Salwanis Binti Wan Md Zain for always being there, guiding, assisting, supporting and reminding. I have learned a lot from her and I would cherish everything that she taught.

I would also like to express my gratitude to Madam Chua for guiding me during the process of my experiment.

Not to forget to all my course mates for being supportive, sharing knowledge and gave me strength to complete this project. I will never forget the times that we have spent together.

Lastly I would like to thank University College of Engineering & Technology Malaysia (UTEC) for providing excellent facilities to make possible of this project. Also, to all the staff in Faculty of Chemical & Natural Resources Engineering, especially the Technical engineers in the lab, your \assistance are deeply appreciated

ABSTRACT

Lignin creates the main problem in waste from the pulp and paper industry. The pulp and paper industry is always interested in finding new, cheaper and more effective ways of get lignin out so that they can get to the cellulose fibres. Lignin degrading bacteria and enzyme is characterized. Important aspects of the production of the enzyme by lignin degrading bacteria studied include the characterization of enzyme and improvement of yield. This process includes a two phases: screening process and the characterization process. Screening process was done using selective agar containing alkaline lignin. The microbe was characterized using gram staining, spore staining and acid-fast staining. The lignin degrading microbe found was tested; the result was a gram-negative bacteria, has no endospore and is not acid fast. The optimum pH found was 6.0 while the optimum temperature examined was 40°C and gave the maximum enzyme activity of 2.00E-^{05} μmol/ml/min. The effect of carbon source and nitrogen source on the enzyme production was also studied. Carbon sources that were compared were carboxy methyl cellulose, glucose and xylose. It was proven that xylose is the best carbon source among the three. While nitrogen sources compared were yeast extract, peptone and urea. The best nitrogen source among the three was yeast extract and peptone.

ABSTRAK

Lignin merupakan punca masalah utama dalam sisa pulpa dalam industri pembuatan kertas. Industri pembuatan kertas berminat untuk mendapatkan cara baru untuk menangani masalah ini. Aspek penting untuk pengeluaran enzim dari bakteria pengurai lignin termasuk pengelasan ciri enzim dan peningkatan pengeluaran. Ia melibatkan dua fasa: proses pengskrinan dan proses pengelasan ciri. Pengskrinan dibuat dengan menggunakan agar pemilih yang hanya mengandungi alkali lignin sebagai sumber karbon. Bakteria yang dijumpai diuji dengan gram staining, spore staining dan acid0fast staining untuk mengetahui ciri-cirinya. Hasilnya, bakteria yang dijumpai merupakan bakteria gram-negatif, tiada endospore dan bukan acid-fast. pH optima didapati adalah 6.0 manakala suhu optima diuji adalah 40°C dan menghasilkan aktiviti enzim tertinggi sebanyak 2.00E-^{05} μmol/ml/min. Kesan sumber karbon dan nitrogen juga diuji untuk mengetahui karbon dan nitrogen terbaik untuk bakteria tersebut. Sumber karbon yang diuji termasuk carboxy methyl selulosa, glukosa and xylose. Xylose merupakan sumber karbon yang terbaik antara ketiga-tiga diuji. Sumber nitrogen yang diuji adalah ekstrak yis, peptone dan urea. Sumber karbon terbaik adalah ekstrak yis dan peptone.

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LIST OF SYMBOLS

µl	-	Microliter
ml	-	Mililiter
°C	-	Degree Celcius
w/v	-	Weight per Volume
%	-	Percentage
g	-	gram

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CHAPTER 1

INTRODUCTION

1.1 Introduction and Problem Statement

Lignin is one of the three components in the woody plant cell wall. When plants die, it will rot and degrade in soil. Soil contains various kinds of microbe produces enzyme that degrades lignin. Research is widely done on fungi that degrade lignin. While bacteria simply grow faster and multiply faster, it is anticipated to be better in production of lignin degrading enzymes.

There are many types of lignin degrading enzymes such as lignin peroxidase, manganase peroxidase, laccase and glyoxal oxidase. While most of these enzymes are produced from fungi, it can also be produced by bacteria such as lignin peroxidase by the bacteria *Streptomyces Viridosporus*. (Ramachandra *et al*,. 1987)

Lignin creates the main problem in waste from the pulp and paper industry. Paper manufacturers traditionally used toxic chemicals like chlorine to separate the stubborn lignin from woody fibers. The pulp and paper industry is always interested in finding new, cheaper and more effective ways of getting that lignin out so that they can get to the cellulose fibers. It is best to benefit both the environment and industry without harming the environment or impeding the industry. While helping the paper industry, there are other potential applications of lignin-degrading microbes and enzymes including; pulping wood, bleaching chemical pulps, improving mechanical pulps, converting lignin to chemicals, and treating lignin-derived wastes. (Research Magazine 1993)

The main purpose of this work is to screen and characterize the bacteria from soil that produce the lignin degrading enzyme.

1.2 Objectives of Study

The objective of this experiment is to screen microbe from soil that produce enzyme that degrades lignin. Besides that, effects of carbon and nitrogen sources were examined to enhance the production of lignin degrading enzyme.

1.3 Scope of Study

The scope of the experiment consists of four parts;

i) Screening of microbe from sample soil

ii) Characterization of the microbe using Morphology tests.

iii) Characterizing the lignin degrading enzyme by studying the effects of pH and temperature on the enzyme.

iv) To study the effect of carbon and nitrogen sources of the production of lignin degrading enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Lignin

Lignin is a chemical compound that is an integral part of the cell walls of some cells, e.g., tracheids, xylary fibres and sclereids of plants.

Lignin macromolecules are mostly made of phenylpropanoid units linked to each other through different kinds of bonds. Lignin macromolecules are probably network structures with molecular weights on the order of 10,000 amu. (Wikipedia.com)

Lignin is the most abundant organic material on earth after cellulose. It is believed that lignin gives wood its stiffness and improves water transport. Lignin is thought to act as a kind of glue in the plant cell walls and give plants very effective protection against parasite attack. Lignin makes up about one-quarter to one-third of the mass of dry wood. In the chemical pulping process, lignin is removed from wood pulp before it is turned into paper, and the extracted lignin is used as a binder in particleboard, adhesive for linoleum, and raw material for processing into chemicals (such as dimethyl sulfoxide and vanillin). The type of lignin (such as lignosulfonates and kraft lignins) used in industry depends upon the method that was used to extract it. (Biocrawler.com)

2.2 Lignin degrading enzymes

Due to the fact that lignin has a complex structure, it is not only degrading by one particular enzyme. Different enzymes may degrade lignin by cleaving different parts of the lignin structure. Table 2.0 shows various types of enzyme and parts of lignin it degrades.

Table 2.0: Enzymes involved in the degradation of lignin and their main reactions (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 enzymes	Many organic compounds	O_2 reduced to H_2O_2

[Annele Hatakka]

2.2.1 Lignin peroxidase

Lignin peroxidase (LiP) is an enzyme that is used to degrade lignin. It was firstly discovered in 1983. Lignin peroxidases are produced by many wood degrading fungi as a family of isoenzymes (Kirk and Farrell, 1987). Recent researches also showed that it can be produced from bacteria such as *Streptomyces Viridosporus*.

These hemeproteins, which are approximately 37,000 daltons in size, are similar to the more familiar plant peroxidases in structure and mechanism, and utilize hydrogen peroxide and organic peroxides to oxidize a variety of substrates (Tien *et al.,* 1986). Some of the most important features distinguishing these enzymes from other oxidoreductases (such as horseradish peroxidase), for example, are their very low pH optima and much higher redox potentials.

The substrates of lignin peroxidase include both phenolic and nonphenolic aromatic compounds; the phenolic substrates are oxidized to yield products similar to those produced by classical peroxidases, while the oxidation of the nonphenolic methoxybenzenes is unique to the lignin peroxidases (Kersten *et al.,* 1985). The oxidation of these substrates to yield aryl cation radicals can result in; demethoxylation, C_a-$C_ß$ cleavage of lignin model compounds, benzylic alcohol oxidation, and hydroxylation of aromatic rings and side chains

The substrate range is very broad, with reactivity are determined by the redox potential. Lignin peroxidase can catalyze the oxidation of substrates with a reduction potential greater than 1.3 volts. The enzyme is capable to oxidize lignin monomers, dimers and trimers as well as polycyclic aromatic compounds such as benzopyrene (Haemmerli *et al.,* 1986).

The powerful and relatively nonspecific nature of this enzyme has lead to investigation of its potential use in diverse fields including specialty chemical synthesis, biodegradation of toxic chemicals, pulp & paper processing, and the textile industry.

2.2.2 Laccases

Laccases are blue copper oxidases that catalyse the one-electron oxidation of phenolics and other electron-rich substrates. Laccases, are commonly produced by ligninolytic fungi *P. chysosporium*. Laccases contain multiple copper atoms which are reduced as the substrates are oxidized. The action of laccase on lignin resembles that of Mn(III) chelates, in that phenolic units are oxidized to phenoxy radicals, which can lead to degradation of some structures (Kawai *et al.,* 1988). In the presence of certain artificial auxiliary substrates, the effect of laccase can be enhanced so that it oxidizes non-phenolic compounds. LiP must therefore be considered an important ligninolytic agent, but it may act in concert with other, smaller oxidants that can penetrate and open up the wood cell wall.

2.3 Lignin degrading bacteria

As mentioned, lignin degrading enzymes may come form both bacteria and fungi. There are several other bacteria that produce other types of enzyme to degrade lignin. For example, *Pseudomonas putida* also produce Vanillate O-demethylase oxidoreductase. While the more common lignin peroxidase and laccase are produced by *Streptomyces viridosporus* and *Bacillus subtilis* respectively.

2.3.1 *Streptomyces viridosporus*

Streptomyces Viridosporus is well known as bacteria that degrade lignin by producing the enzyme lignin peroxidase. It belongs to the group Actinobacteria or more commonly called Actinomycetes (Gram-positive Bacteria). It is usually found in soils where lignin is present.

2.3.2 *Bacillus subtilis*

This organism was one of the first bacteria studied, and was named *Vibrio subtilis* in 1835 and renamed *Bacillus subtilis* in 1872. It is one of the most well characterized bacterial organisms, and is a model system for cell differentiation and development. This soil bacterium can divide asymmetrically, producing an endospore that is resistant to environmental factors such as heat, acid, and salt, and which can persist in the environment for long periods of time. The endospore is formed at times of nutritional stress, allowing the organism to persist in the environment until conditions become favorable. Prior to the decision to produce the spore the bacterium might become motile, through the production of flagella, and also take up DNA from the environment through the competence system.

2.3.3 *Pseudomonas Putida*

Pseudomonas putida is a bacterium that is flagellated rod-shaped. It is known for it's ability to degrade organic solvents. These solvents include toluene, in gasoline. This ability has been put to use in bioremediation, or the use of microorganisms to biodegrade oil. *P. putida* is a safe strain of bacteria which is not pathogenic, unlike *P. aeruginosa*, a human pathogen.

2.3.4 *Pseudomonas Paucimobilis*

Pseudomonas paucimobilis is a new species isolated from human clinical specimens, the hospital environment, and other sources. It has the ability to degrade various dimeric lignin compounds (Y. Katayama *et al.*, 1987). As all pseudomonas members, *Pseudomonas paucimobilis* is also gram-negative.

2.3.5 *Pseudomonas flourescens*

Pseudomonas fluorescens is a common Gram-negative, rod-shaped bacterium . It secretes a soluble fluorescent pigment called fluorescein. *P. fluorescens* also has multiple flagella. It has extreme versatile metabolism, and can easily be found in soil and in water. It is an obligate aerobe but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration.

Optimal temperatures for growth of Pseudomonas fluorescens is around the temperature of 25-30°C. It results positive for the oxidase test.

Heat stable lipases and proteases are produced by *Pseudomonas fluorescens* and other similar pseudomonads(Frank 1997). These enzymes cause milk to spoil, by causing bitterness, casein breakdown, and ropiness due to production of slime and coagulations of proteins (Jay 2000, Frank 1997, Ray 1996).

2.4 Possible lignin degrading organisms and enzymes

Actinomycetes are gram positive bacterial found in large of habitats like soil, composts and decaying plants. And hemicelluloses represent important substrates in theses habitats, actinomycetes can be considered as a potential source of hemicellulolytic enzyme. Streptomyces bacterial are gram positive and soil inhabiting. (Hiroshi *et al.,* 2004) *Streptomycetes* grew most in pore spaces which were humid and air-filled, and growth was reduced in water-logged pores. Spores of *Streptomycetes* survived for long periods in dry soil. (Williams *et al.,* 1972) Table 1.1 show some of the potential lignin degrading enzyme producers by various authors.

Table 2.1 Example microorganisms that produces lignin degrading enzyme

Microorganisms	Enzyme	Optimum Condition	Optimum Activity	References
Streptomyces viridosporus T7A	Lignin peroxidase	pH 7.5 to 8.5	350 U/L	Macedo *et al*., 1999
Pseudomonas Paucimobolis	C alpha-dehydrogenase	N/A	N/A	N/A
Pseodomonas putida	Beta-etherase	30°C	N/A	Steddom *et al*., 2002
	Protein ligF			
	Vanillate O-demethylase oxidoreductase			
Bacillus subtilis	Laccase	30°C to 37 °C	N/A	Korsten and Cook, 1996

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

All of the chemicals used in this experiment were of analytical grade. Commercial lignin, carboxy methyl cellulose, glucose, yeast extract, peptone, and urea were obtained from Fluka (Aldrich Chemical).

3.2 Culture Conditions for Lignin Degrading Microbes

3.2.1 Source of lignin degrading microbe

Sample soil containing lignin degrading microbe was taken from forest located around KUKTEM campus. Sample soil was taken 5cm from the surface of the soil. Sample soil is kept from contamination and stored in room temperature. The microbe in soil was screened and was used for subsequent works.

3.2.2 Storage of microbes

The microbes screened were stored in glycerol stock in -80°C.

3.3 Medium

3.3.1 Agar Medium

Agar medium is considered as solid media as gelatin or silica gel. It serves as food for culturing bacteria. This agar supports all types of bacteria. It usually comprise of water, carbon and minerals. A total of 0.2% (w/v) nutrient agar was used.

3.3.2 Seed Culture Medium

The selective agar comprise of lignin as the main component and minerals to support the growth of lignin degrading microbe.

The medium consists of 0.4 % (w/v) alkaline lignin, 0.4 % (w/v) yeast extract, 1.0 % (w/v) malt extract, and 0.2 % (w/v) calcium carbonate.

Microbe that grows on the selective agar was transferred weekly for 5 times to ensure strain of bacteria is pure enough for morphology and physiological tests.

3.3.3 Production Medium

Several carbon sources were selected to compare amongst each other to find out which may become the most economical and efficient in production. Production medium comprise of lignin and minerals that supports the desired microbe.

The production medium composition was according to the seed culture medium, only by replacing the alkaline lignin with other carbon source such as carboxy methyl cellulose, glucose and xylose. When the best carbon source was selected, the best nitrogen source was tested. The composition of the medium is 0.4 % (w/v) carbon source and 0.4 % (w/v) nitrogen source.

3.3.4 Preparation of Inoculum

A loopful of microbe from the afar medium was transferred to the 250 ml shake flask containing 30 ml of seed culture medium. The culture was incubated at 33°C for 18 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 600nm using spectrometer until it reaches 0.5.

This process was done before the incubation process of determining the effects of carbon source and nitrogen source.

3.5.5 Production of Crude Enzyme

10% v/v of bacteria inoculums was transferred to 250ml shake flask continuing production medium, giving the total volume of 30ml. 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.

3.4 Characterization of Microbes

3.4.1 Morphology Test

3.4.1.1 Gram Staining

Gram staining technique was used to determine the structure of the microbe. The selected microbe that grows on lignin was tested whether it is gram positive or gram negative.

Bacteria were smeared on staining rack and slide was stain with crystal violet for 1 to 2 minutes. The slide is then flooded with iodine for another 1 to 2 minutes. The iodine is then poured off. The process was followed by decolorized process using acetone for 2 to 3 seconds, and immediately washed with water. The slide flooded with safranin counterstain for 2 minutes and washed with water. Blot dry with bibulous paper and air dry was examined under the oil immersion.

Gram-positive bacteria are not decolorize by the decolorization process and remain purple. Gram-negative bacteria on the other hand will be counterstained by safranin which results in pink color.

3.4.1.2 Spore Staining

It is known that gram staining will not stain endospores. Thus the spore staining method is used. The microbe was tested of its vegetative spores.

` Using antiseptic technique, screened microbe was smeared into a clean slide. Slide with microbe was then air dried. The slide was then covered with paper towel, placed on staining rack and over a boiling water bath. Paper towel with slide was then flooded with malachite green and it was steamed for 5 minutes. After 5 minutes, the slide was removed, and the paper towel was removed. The slide was left to cool and rinsed with deionized water. Safranin was rinsed off and the slide is blot dry with bibulous paper and examined under oil immersion.

Bacteria which have endospore will result in green and pink. The green color structure is the endospore while the pink color structure is the vegetative cell. Negative result will only show pink color structure of vegetative cell only.

3.4.1.3 Acid Fast Staining

Microbe was tested with Acid-fast staining to check the presence of pathogenic mycobacteria.

Microbe that was smeared and covered with carbolfuchsin, and placed put on boiling hot bath for 5 minutes. Then, the slide is cooled and decolorized using acid-alcohol for 15 to 20 seconds. The decolorization was stopped by rinsing with water. The slide was then counterstained with methylene blue for 30 seconds. Excess methylene blue was rinsed with water. Slide was blot dry with bibulous paper, and examined under oil immersion.

3.5 Physiology Study of Enzyme Characteristic

3.5.1 Effect of pH

Hydrogen ion concentration of an organism's environment exerts a great influence on microbial growth. Concentration of hydrogen ions limits the activity of enzyme.

Through enzyme assay, crude enzyme is being harvested and being tested for the effect of pH. Crude enzyme was being tested by using buffer of 5.0, 6.0, and 7.0 together with substrate. Buffer was prepared by using sodium acetate and acetic acid. Results of the enzyme efficiency under these pH conditions were tested using DNS method.

Crude enzyme under different pH conditions will have different reaction rate thus may cleave to produce more or less simple sugars. By using DNS method, the amount of simple sugar produced by the enzyme under pH conditions can be tested.

3.5.2 Effect of Temperature

In this part, it is mainly to compare the susceptibility of different enzyme to elevated temperatures. A range of temperature that is most suitable for the enzyme was found.

The temperature range examined includes 30°C, 40°C, 50°C, and 60°C. After the optimum pH for the enzyme has been known, further tests on the effect of temperature are being done under the optimum pH. Buffer of the optimum pH is added to substrate to get 0.5% (w/v) and being incubated with 0.1ml crude enzyme. Incubation is done for 15 minutes for each temperature respectively, and the mixture is being tested under DNS method to check the total glucose produced. The highest value of glucose represents the best temperature for the enzyme.

3.6 Physiological Study of Enzyme Characteristics

3.6.1 Enzyme assay

Assay for lignin peroxidase were performed using substrate compose of 0.5% (w/v) alkaline lignin dissolved in 40ml of 0.1 M phosphate buffer with pH of 7.0

After incubation of at 33°C at 200 rpm for 24 hours, the broth is transferred into vial for centrifugation. Centrifugation was done at 5000 rpm for 5 minutes. The supernatant obtained was used as crude enzyme.

0.1 ml of crude enzyme was incubated with substrate for 15 minutes at 33°C. The reaction mixtures were tested for its simple sugar using DNS method.

3.6.2 DNS Method

The Dinitrosalicylic colorimetric method is a procedure to test the existence of simple sugar. This method works by examining the presence of free carbonyl

group (C=O), reducing sugars. This involves the oxidation of the aldehyde functional group that is present. Both glucose and fructose has this functional group. The DNS is reduced to 3-amino, 5-nitrosalicylic acid under alkaline conditions. Different reducing sugars will produce different color intensities. For glucose, a standard curve for glucose itself has to be calibrated.

For the DNS assay procedure, a total of 20 µl of concentrate sulphuric acid is added to the sample solution. Mixture was being through hydrolysis at 90°C for 5 minutes. 0.05 ml of 5N potassium hydroxide solution was added to neutralize the acid. The DNS reagent is added. A calibration curve was generated to correlate the absorbance to the sample.

To test the crude enzyme from enzyme assay, a total of 0.1ml crude enzyme is mixed with 0.5ml substrate. Crude enzyme will react with the substrate added producing simple sugar. The reaction between enzyme and substrate is stopped by using ice bath. After the reaction being stopped, a total of 0.6ml DNS (ratio 1:1 between DNS and total volume of both crude enzyme and substrate) is being added into the solution. Another 60 µl of 0.1N sodium hydroxide is added into the solution. The solution is heated at 100°C for 5 minutes in water bath. After heating, the solution was brought into ice bath to cool.

A glucose standard curve will be calibrated by using different concentration of glucose, and have it's absorbance checked. Absorbance is plotted against glucose; the plotted graph is the glucose standard curve. The calibrated standard curve will be used to determine glucose concentration at various absorbances. The range of the curve is between 1.5 E^{-6} to 8.0 E^{-5} mg/ml of glucose.

3.6.3 Protein Content

Modified Lowry Protein Assay method is used in determining protein content. It uses bovine serum albumin as a standard determined total protein concentration and absorbance. This assay uses three reagents; Reagent A (Sodium Potassium

Tartrate with Sodium Carbonate), Reagent B (Sodium Potassium Tartrate with Copper Sulphate) and Reagent C (Folin-Ciocalteau).

Samples are diluted into 0.25mg/mL with buffer. 400 µl of each dilution was prepared and duplicated. Reference buffer of 400 µl was also prepared. 0.25 mg/mL of bovine serum albumin was prepared. The reference was brought up to 400 µl. 400 µl of Lowry concentrate was added and mixed thoroughly. The mixture was incubated at room temperature for 10 minutes. 200 µl of 0.2 N Folin is added immediately and the sample was incubated for 30 minutes at room temperature. The absorbancy of each sample was measured at 650nm. A standard curve was prepared by plotting the average blank corrected at 650 nm reading for each BSA standard versus its concentration in µl /mL. The protein concentration was determined for each unknown sample using the standard curve.

A Lowry standard curve will be calibrated by using different concentration of protein, and have it's absorbance checked. Absorbance is plotted against protein concentration; the plotted graph is the Lowry standard curve. The calibrated standard curve will be used to determine protein concentration at various absorbances.

3.7 Production of enzyme

3.7.1 Effect of Carbon sources

A variety of carbon sources were used to determine the best carbon sources that enhance lignin peroxidase production. The selection was done using CMC, glucose and xylose.

When the densities of cells are standardized, it is then transferred into the flasks of carbon sources to check which the best carbon source for the microbe is. Medium prepared is the same composition as the seed culture medium, only differ in the carbon source where 0.4 % (w/v) lignin is replaced with the carbon source tested. Incubation is run at 33°C and 200 rpm for 24 hours. After incubation, the sample is being tested of its content of protein released using the Lowry method as described.

The highest protein content is the best carbon source for the founded microbe.

3.7.2 Effect of Nitrogen sources

After the selection of the best carbon source that gives the best increment in lignin peroxidase were made, it was continued with the study of the best nitrogen source that support the growth and lignin peroxidase production. Nitrogen sources tested were yeast extract, peptone, and urea.

Solution of cultures is made according to the best carbon source found. The best carbon source is used while changing the other nitrogen source. Three different nitrogen sources is tested following the incubation process and result testing process as described while finding the best carbon source.

Composition of the medium is the same as the seed culture medium only to differ in replacing 0.4% of alkaline lignin with the best carbon source found, and replacing the 1.4% nitrogen sources with each nitrogen sources to be examined respectively.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Lignin degrading microbe

Sample soil was brought back from forest was diluted for screening process. The dilution process was made 9 times. The 6 times dilution made still contains too many colonies when being transferred into agar plate for incubation. Each dilution dilutes the sample as much as 10^{-1}. The 8^{th} dilution was taken for screening purpose. This is because the 8^{th} dilution agar plate has the clearest sets of colonies.

Colonies from nutrient agar plates was transferred into selective agar plates. A total of 15 colonies from the nutrient agar were transferred into 15 different selective agar plates. Out of 15 selective agar plates, only 6 of them grow. Those were prospective lignin degrading microbes growing. The microbe growing in the plate is assumed to be the lignin degrading microbe since the only carbon source available was only lignin. Only lignin degrading microbe is able to consume lignin as carbon source.

Figure 4.0 Lignin degrading microbe growing on selective agar plate

It was clear that the dark colored agar become less dark around the streaked microbe. It was assumed that lignin (dark color) is consumed by the microbe which grows on the plate.

These plates were transferred into new selective agar plates weekly. This is made for two main reasons which are;

i) To keep the microbe in a condition that there are still carbon, nitrogen, and mineral sources around.

ii) To get purer strain of the microbe found.

This process was done for 5 weeks. This is due to the fact that the first 4 weeks, the four plates transferred, all resulting in two colors during gram staining test. The impurity of the colony causes the stain to result in two colors; that is pink and purple, which shows there were both gram positive and gram negative microbes present. After the 5^{th} week, the strain is considered to be pure since the morphology test does not result in two colors after the 5^{th} transfer. The resulted shape is similar to staphylococci.

The microbe was kept in an incubator at 33°C. For enzyme characterization, microbe is transferred into shake flask and being harvested through enzyme assay.

Shake flasks of 6 different microbes from their agar plates respectively was incubated. 5 of them were being identified as fungi (identified visually – forming of flocculants). The remaining flask is identified as bacteria since it shows precipitation.

Shake flask of fungi was not being taken into test because bacterial strain is the more preferred microbe.

4.2 Morphology tests results

4.2.1 Gram staining results

Gram staining was done on the potential lignin degrading microbe. Microbe was put into slide and stained as gram staining process described. Figure 4.1 below shows the result of gram staining under microscopic view.

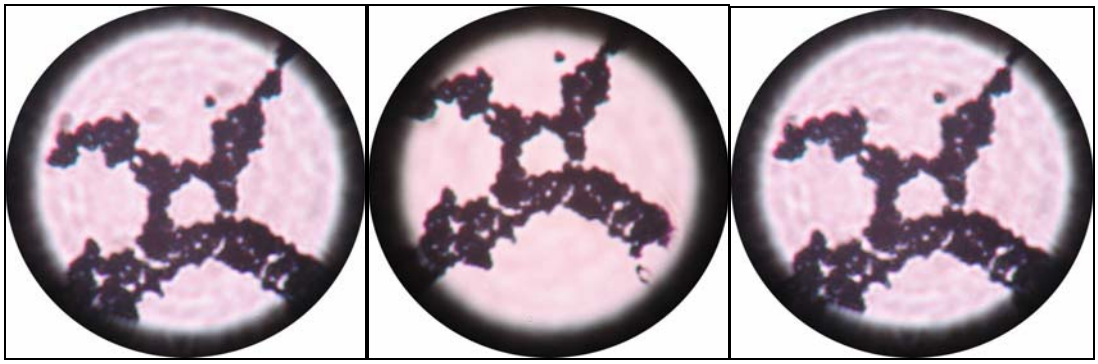

Figure 4.1 Gram staining slide under microscopic view

From the result, it is observed that the bacteria stain were pink in colour. This shows that the microbe were a gram-negative bacterium.

4.2.2 Spore staining results

The microbe was also tested for its endospore structure. The microbe was stained and the results are as the pictures below:

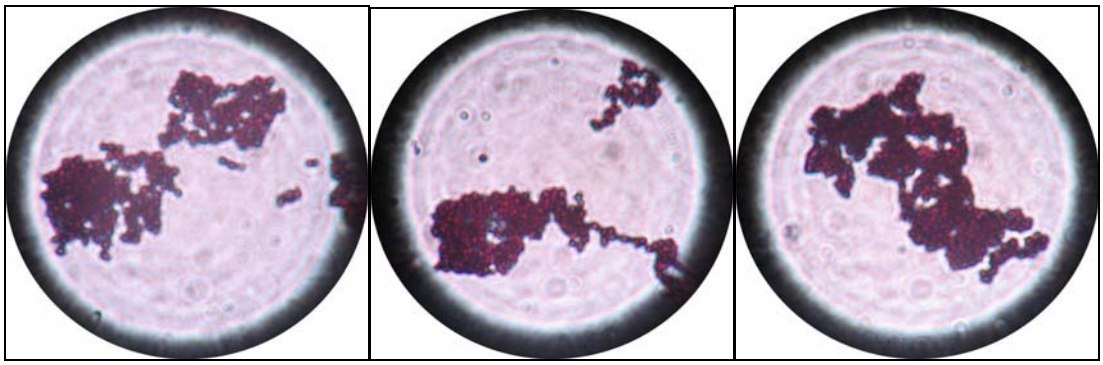

Figure 4.2 Spore staining slide under microscopic view

From the figure 4.2 above, it shows that stain only result in pink colour. There were no green vegetative cells shown after the staining. From this, it is assumed that it was not from *bacillus* and *clostidium*.

4.2.3 Acid-fast staining results

Acid-fast staining was done of the selected microbe. The results are as follow;

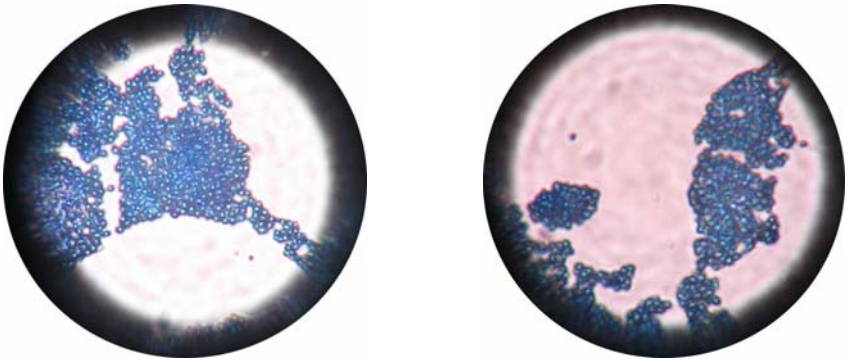

Figure 4.3 Acid-fast staining slide under microscopic view

Figure 4.3 shows that the bacteria do not tend to be acid-fast. This removes the possibility of the microbe being a *mycobacterium*. (Benson 1994)

The resulting staining results may be similar to lignin degrading pseudomonas strains of 109, and 170 as researched by Tserovska. (Tserovska *et al.*,2004).

4.3 The effect of pH and Temperature on Enzyme

4.3.1 The effect of pH

Crude enzyme was being tested for its optimum pH. The following is the results of DNS tests after crude enzyme of are being tested with lignin substrate at each pH respectively.

Table 4.1 Effect of pH on enzyme activity

pH	ABS	Glucose Concentration (mg/ml)	Enzyme Activity, U (μmol/ml/min)
5.0	1.710	4.34054E-05	1.61E-05
6.0	2.131	5.40918E-05	2.00E-05
7.0	2.081	5.28226E-05	1.96E-05

According to the standard curve of simple sugar (which is optical density is proportional to absorbance), the higher the absorbance shows the higher amount of simple sugar exists in the solution. This shows that; at pH 6.0, the enzyme functions at optimum condition and produce the most number of simple sugars.

The resulting pH of 6.0 is probable due to the original sample taken was at a local area where acid rain is common. There are also lignin degrading enzymes that have an optimum pH of 6.0 such as Aryletherase that degrade aromatic rings. (Srinivasan *et al.,* 1987)

4.3.2 The effect of Temperature

Crude enzyme was again being test but for the optimum temperature. A series of temperature is being tested by incubating the sample with lignin substrate under the optimum pH of 6.0. The result of incubation is being tested for its absorbance and the results are as the following;

Table 4.2 Effect of temperature on enzyme activity

Temperature	ABS	Glucose Concentration (mg/ml)	Enzyme Activity, U (μmol/ml/min)
30°C	1.767	4.48523E-05	1.66E-05
40°C	1.933	4.90659E-05	1.82E-05
50°C	1.392	3.53335E-05	1.31E-05
60°C	1.375	3.4902E-05	1.29E-05

From the results, the temperature that records the highest absorbance is 40°C. This shows that lignin degrading enzyme's optimum temperature is when it is 40°C. This is most possible because the microbe's original environment, sample soil was taken from local area with temperature ranging from 21-33°C. A more precise optimum temperature may be determined if test parameters were to run at smaller deviation such as increment of 2°C.

4.4 Effect of carbon sources and nitrogen sources

4.4.1 Effect of carbon source

Three different carbon sources (carboxy methyl cellulose, xylose and glucose), is being tested after incubation at 33°C, 200 rpm for 24 hours. The result is tested using Lowry method. This is to test the amount of enzyme released after one day incubation. The higher amount of enzyme released means that the carbon source used is better.

After Incubation, 1ml of sample is being taken out from the culture and being transferred into test tubes and Lowry assay is being performed. The result of absorbance is as table 4.3 below:

Table 4.3 Results of carbon sources, absorbance and protein concentration

Carbon Source	Absorbance	Protein Concentration (mg/ml)
Carboxy Methyl Cellulose	1.418	14.52991
Xylose	2.244	24.61538
Glucose	1.437	14.7619