

**LIGNIN DEGRADATION OF BANANA STEM WASTES
USING Aerobic *Bacillus cereus***

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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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I declare that this thesis entitled “Lignin degradation of banana stem wastes using Aerobic *Bacillus cereus*” 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 : 30 April 2009

*Special Dedication to my family members,
my friends, my fellow colleague
and all faculty members*

For all your care, support and believe in me.

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ABSTRACT

The delignification process of banana stem wastes using *Bacillus cereus* was studied. This study is divided into three parts which are determination growth curve of *Bacillus cereus*, batch fermentation and lignin analysis using Klason method. Delignification process is functioning to degrade lignin from banana stem to get cellulose that can be used as carbon sources. *Bacillus cereus* is used as microorganisms to degrade lignin from the banana stem wastes. The banana stem contain 35% of cellulose on dry weight basis. The methods are divided into three parts which are preparation of raw material and microorganisms, fermentation process and lignin analysis. Fermentation is carried out aerobically at 30°C and 37°C, and 250 rpm of agitation speed for 2 days. In this process, Klason method is used to identify the percentage of lignin degraded. Based on the analysis, it shows that *Bacillus cereus* is able to degrade lignin from banana stem wastes. The optimum temperature for *Bacillus cereus* is 37°C with the average percentage of lignin degradation is 28.69%.

ABSTRAK

Proses pengurangan lignin dalam batang pisang menggunakan *Bacillus cereus* telah dikaji. Kajian ini dibahagikan kepada tiga bahagian, iaitu penentuan graf pertumbuhan bagi *Bacillus cereus*, proses fermentasi, dan analisis lignin menggunakan kaedah Klason. Proses pengurangan lignin bertujuan untuk mengurangkan lignin dalam batang pisang supaya kandungan selulosa di dalamnya boleh digunakan sebagai sumber karbon. *Bacillus cereus* adalah salah satu mikroorganisma yang digunakan untuk tujuan ini. Sebanyak 35% selulosa terkandung di dalam batang pisang dalam keadaan berat kering. Prosedur bagi kajian ini terbahagi kepada tiga bahagian iaitu penyediaan bahan mentah dan mikroorganisma, proses fermentasi, dan analisis lignin. Proses fermentasi dijalankan secara aerobik pada suhu 30°C dan 37 °C, dengan kelajuan putaran 250 rpm selama 2 hari. Kaedah Klason digunakan untuk mengenalpasti peratusan pengurangan lignin. Hasil analisis menunjukkan *Bacillus cereus* berkeupayaan untuk mengurangkan lignin dalam batang pisang. Suhu optimum bagi pengurangan lignin dalam keadaan aerobik ialah 37 °C dengan purata peratusan pengurangan lignin sebanyak 28.69%.

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LIST OF SYMBOLS/ABBREVIATIONS

ATP	= adenosine 5'-triphosphate
C	= carbon
Ca ²⁺	= ion calcium
g	= gram
hr	= hour
H ₂ O ₂	= hydrogen peroxide
kDa	= kilo Dalton
ml	= mililitre
Mn ²⁺ and Mn ³⁺	= ion manganese
N	= nitrogen
NAD ⁺	= nicotinamide adenine dinucleotide
OD	= optical density
S ⁺	= ion sulphur
µm	= micrometer
°C	= degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Plastics are polymer which is a compound of high molecular weight and the structure of which is composed of chains of small repeat units. Plastics are composed primarily of carbon, hydrogen and oxygen. Initially, plastics had been made from the use of natural materials (e.g., chewing gum, shellac). In the nineteenth century, Charles Goodyear discovered vulcanization of rubber (1839) and Alexander Parkes, English inventor (1813-1890) created the earliest form of plastic in 1855 which are based on chemically modified natural polymers. Alexander Parkes mixed pyroxylin, a partially nitrated form of cellulose (cellulose is the major component of plant cell walls), with alcohol and camphor. It is known as "Parkesine", a hard but flexible transparent material. Then, the first plastic based on a synthetic polymer was made from phenol and formaldehyde, with the first viable and cheap synthesis methods invented by Leo Hendrik Baekeland in 1909 and known as *Bakelite*. Nowadays, poly(vinyl chloride), polystyrene, polyethylene (polyethene), polypropylene (polypropene), polyamides (nylons), polyesters, acrylics, silicones, polyurethanes were among the many varieties of plastics developed and mainly made from one major ingredient; oil.

Synthetic polymers are easily molded into complex shapes, have high chemical resistance, more or less elastic and some can be formed into fibers or thin transparent films. They have molecular weight ranging from several thousands to 1 500 000. This excessive molecular weight had made them resistance to

biodegradation and their persistence in soil environment for a long time. Plastic in the environment is regarded to be more an aesthetic nuisance than a hazard, since the material is biologically quite inert. Plastics have contributed to pollution and loss of environmental quality. In the United State (US), the plastic industry alone is \$ 50 billion per year and 20% of solid municipal wastes are plastic. In 1999 there are more than 100 million tonnes of plastic is produced every year all over the world. In India use of plastic is 2 kg per person per year while in European countries it is 60 kg per person per year and in US it is 80 kg per person per year.

Plastics have known as one of the caused of the pollution. Therefore, the plastics itself had been altered. Plastics may be either photodegradable or semi-biodegradable or 100% biodegradable. Photodegradable plastics have light sensitive groups incorporated directly into backbone of the polymer as additives. This produces non-degradable smaller fragments, which cause loss of material integrity. Example of semi-biodegradable plastic can be blends of starch and polyethylene. Poly b - hydroxy butyrate (PHB) is an example of 100% biodegradable plastic (Vert *et al.*, 1992). There are three approaches that applied which are modification of existing material, chemical co-polymerization of known biodegradable material and use of biopolymers for making plastics. Biopolymers or also known as bioplastics is an alternative to overcome the problem caused by plastics. Biopolymers obtained from growth of microorganisms or from plants which are genetically-engineered to produce such polymers are likely to replace currently used plastics at least in some of the fields. PHB and polylactic acid are the kind of polymers which are used as materials of bioplastics. Bioplastics are also can be describe as a form of plastics derived from natural resources such as wood (cellulose), vegetable oils, sugar or starch.

PHB is a widespread storage material in many microorganisms like *Alcaligenes*, *Azotobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, *Rhizobium* etc. PHB has physical properties comparable with polypropylene (PP). Fermentation process by those microorganisms will produce PHB. The main nutrients for them are carbohydrates such as sucrose or glucose (as carbon source). Organic plant wastes such as oil palm, pineapple, banana stem and coconut fiber can produce a large

quantities of cellulose and non-cellulose raw material. In Malaysia, a large area of plantation of banana (34 thousands ha) will enable to produce large amount of cellulose that will be used as carbon source for microorganisms in fermentation process. This will involve delignification process by using microorganisms to extract cellulose.

1.2 Objective

The aim of this study is to extract cellulose from banana stem waste that will be used as nutrient (carbon source) for the microorganisms producing PHB. Hence, the objective is

- To study the submerged fermentation of banana stem waste using *Bacillus cereus*
- To study the ability of *Bacillus cereus* in delignify the banana stem waste

1.3 Scope of Study

Based on the objectives, the scopes of study are highlighted as follows:

- Submerged fermentation is used as fermentation condition for delignification of banana stem waste using *Bacillus cereus*
- Test the lignin content of delignification of banana stem waste by *Bacillus cereus*

1.4 Problems Statement

Due to the effect that had caused by the conventional plastics, bioplastic had become the other alternatives. In early development of bioplastic, the cost for the

production is high compared to the conventional plastic. In 1982 ICI, the UK chemical group, has opened a plant at Billingham in North-east of England to make 300 tonnes of bioplastic a year, which it says is the first fully biodegradable commercial plastic. At present, bioplastic costs about 10 pounds per kg., 20 times more than conventional plastic (Lee S.Y, 1996). To reduce the cost, organic plant wastes are used as the raw materials for the carbon source. By using plants, cost can be reduced tenfold.

In Malaysia, banana had been produced in a large quantity which is the total plantation area is 33 704.2 hectares (Abdul Khalil *et al.*, 2006). Banana consists of stem that call as pseudostem. After harvesting, those pseudostems are lefted waiting to be degraded naturally. According to Abdul Khalil *et al.*, (2006), the content of cellulose is high (> 40%) in pseudostem. However, those pseudostems have high content of lignin. Lignin provides plant tissue and individual fiber with compressive strength and stiffens the cell wall of the fibers, to protect the carbohydrates from chemical and physical damage (Saheb and Jog, 1999). Lignin is an undesirable polymer, and its removal during pulping requires high amount of energy and chemicals (Abdul Khalil *et al.*, 2006). Therefore, delignification had taken place to delignify the pseudostem. The delignification process is carried out using microorganisms. Therefore it is important to determine the effectiveness of microorganisms such as *Bacillus cereus* in delignification process.

1.5 Rationale & Significance

The main purpose is to produce bioplastic that is environmental friendly. By using the organic plant wastes like banana stem (as raw materials) to produce cellulose (as carbon source) it is indirectly prevented another caused of pollution. Besides that, it is to prove that all the component of banana plant itself is useful including the stem. Other than that, wastes can play the role as carbon source instead of using food materials. The cost for the production of bioplastic will be decrease when using wastes as the source of the carbon source for PHB production.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Products base on plastic are widely used over the world. The highly demand of plastic had cause problem to the environment because it is not biodegradable. When the Poly b-hydroxy butyrate (PHB) had been described in 1926 by Lemoigne which has similar properties with polypropylene (PP), numerous researches had been done in producing PHB polymer. PHB polymer is material of bioplastics that are biodegradable. Many countries have come out with research for production of PHB. W. R. Grace was the American company which carried out the work on PHB as early as 1960s. The development of PHB was begun by ICI in 1975-6 as a response to increase in oil prices. Production of PHB from *Alcaligenes eutrophus (H16)* by a subsidiary of ICI Ltd. in Great Britain, production of PHB in transgenic plants that was described by MIT researchers in a 1989 patent application and production of PHB in genetically engineered bacteria by Metabolix researchers are some of the methods that had been applied (Lee *et al.*, 1996).

Most of the productions of PHB are dealing with microorganisms. It is seem to be highly cost compared to the conventional plastics. To cut the cost, the source of carbon source that used as nutrients to microorganisms are becomes an important part. Organic palm wastes such as banana stem are being considered. This type of raw materials needs to undergo delignification process because the stem is covered with the lignin that protects carbohydrates from chemical and physical damage.

2.2 Plastic and Bioplastic

Plastic is the general common term for a wide range of synthetic or semisynthetic organic solid materials suitable for the manufacture of industrial products. Plastics are typically polymers of high molecular weight, and may contain other substances to improve performance or reduce costs (Wikipedia, 2008). The word derives from the Greek πλαστικός (plastikos), "fit for molding", from πλαστός (plastos) "molded". It refers to their malleability or plasticity during manufacture, that allow them to be cast, pressed, or extruded into an enormous variety of shapes—such as films, fibers, plates, tubes, bottles, boxes, and much more. The word is also commonly used an adjective with the sense of "made of plastic" (e.g. "plastic cup", "plastic tubing") (Wikipedia, 2008).

2.2.1 Structure of polymer

Most of the polymers consist of hydrocarbon. These polymers are specifically made of small units bonded into long chains. Carbon makes up the backbone of the molecule and hydrogen atoms are bonded along the backbone (Americanchemistry, 2007). The basic make up of polymer is carbon and hydrogen. However other elements can also be involved such as oxygen, chlorine, fluorine, nitrogen, silicon, phosphorous, and sulfur.

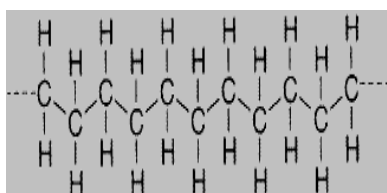


Figure 2.1: Structure of polyethylene, the simplest polymer structure

2.2.2 Classification

Plastics can be classified by their chemical structure, namely the molecular units that make up the polymer's backbone and side chains or based on qualities that are relevant for manufacturing or product design. Plastics can also be ranked by various physical properties, such as density, tensile strength, glass transition temperature and resistance to various chemical products. Plastics are used in various range of product and already displaced many traditional materials such as wood, stone and ceramic in most of their former uses. This is because plastics have relatively low cost, ease of manufacture, versatility, and imperviousness to water

2.2.3 Development of plastic

The development of plastics has come from the use of natural plastic materials (e.g., chewing gum, shellac) to the use of chemically modified natural materials (e.g., rubber, nitrocellulose, collagen, galalite) and finally to completely synthetic molecules (e.g., bakelite, epoxy, polyvinyl chloride, polyethylene) (Wikipedia, 2008). The first man-made plastic was created by Alexander Parkes who publicly demonstrated it at the 1862 Great International Exhibition in London. The material called Parkesine was an organic material derived from cellulose that once heated could be molded, and retained its shape when cooled (About.com, 2008)

Due to their insolubility in water and relative chemical inertness, pure plastics generally have low toxicity in their finished state, and will pass through the digestive system with no ill effect. Plastics are often containing a variety of toxic additives. For example, plasticizers like adipates and phthalates are often added to brittle plastics like polyvinyl chloride (PVC). Plastics are durable and degrade very slowly. In some cases, burning plastic can release toxic fumes. Also, the manufacturing of plastics often creates large quantities of chemical pollutants (Wikipedia, 2008)

To overcome the pollution problem, researches had been done in order to produce plastic that is biodegradable. Therefore bioplastic which is obtain from growth of microorganisms or from plants which are genetically-engineered to produce such polymers are likely to replace currently used plastics at least in some of the fields. Poly b - hydroxy butyrate and polylactic acid are the kind of polymers which are used as materials of bioplastics.

2.3 Raw Materials for Carbon Source

Any materials that contain carbohydrates will be use as a raw material to produce carbon source. Organic palm waste has preferred because of economical aspect. Banana is the common name for a fruit and also the herbaceous plants of the genus *Musa* which produce this commonly eaten fruit (Wikipedia, 2008). Bananas are produced in large quantities in tropical and subtropical area. Banana plants range in height from 0.8 m to more than 15 m (Abdul Khalil *et al.*, 2006). Each contains a flattened, modified stem, called a pseudostem, consisting of concentric layers of leaf sheath and crown of large leaves (Ennos *et al.*, 2000). They are cultivated primarily for their fruit, and to a lesser extent for the production of fibre and as ornamental plants (Wikipedia, 2008). This means that the pseudostem will be wasted, for every 60 Kg of banana grown; 200 Kg of waste stem is thrown away. According to Prof. Morishima, annually one billion tonnes of banana stem are left to rot in the world (Wirefabrik, 2004). Currently these wastes are dumped in landfills, rivers, oceans and unregulated dumping grounds. When they reach these destinations, they form huge masses of putrefying wastes that attract insects and scavengers, spread disease, contaminate groundwater, and have foul odors that can be smelled for miles around (Leon, 1997)

Effective Microorganisms (EM) are currently being used in many countries as a beneficial microbial inoculate for processing organic materials so that they can be recycled back into agricultural systems (Afzal *et al.*, 1994). Experiments were carried out in Costa Rica using EM to process all forms of banana wastes into rich amendments that can improve soil structure and increase crop yields and quality. The

products made using wastes from the banana industry can be used as beneficial microbial inoculates for soil regeneration and fertilization (Higa, 1996). Apart than that, microorganisms are also can be used to degrade banana stem. Banana stem contains lignin 22% and cellulose 35% on dry weight basis (Saha *et al.*, 1999). Banana are also has very high moisture content; 83% of its solids are volatile which offer great potential for anaerobic digestion. The cellulose contain will be used as carbon source to produce PHB, material for bioplastics production.

2.4 Lignin and Delignification process

Plant cell wall is composing of cellulose, hemicellulose and lignin. Lignin is a complex chemical compound that had played a crucial part in conducting water in plant stems. Lignin is formed through oxidation and free radical coupling of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. The cross-link of polysaccharides by lignin had become an obstacle for water absorption to the cell wall. This will make the plant's vascular tissue to conduct water effectively because the polysaccharides component of cell wall is hydrophilic (permeable to water) while the lignin is hydrophobic.

Lignin is a formidable substrate (Higuchi, 1990; Lewis and Sarkanen, 1998) thus makes it difficult to degrade. Mammalian and other animal enzymes are unable to degrade the lignin. However, some fungi and bacteria are able to biodegrade the lignin. Lignin degradation requires oxidative attack on the carbon-carbon and ether interunit bonds. Degradation of lignin will enable to gain access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources. Lignolytic enzymes such as manganese peroxidase, laccase and cellobiose dehydrogenase are some example of microbial enzyme that involved in the lignin degradation.

Previous research shown that white-rot fungus *Phanerochaete chrysosporium* is enable to degrade lignin. The enzymes from white rot fungi that catalyze the initial depolymerization of lignin are extracellular and unusually nonspecific (R. Brambl

and G.A. Marzluf, 2004). A constellation of oxidases, peroxidases, and hydrogen peroxide are responsible for generating highly reactive free radicals that undergo a complex series of spontaneous cleavage reactions. The nonspecific nature and extraordinary oxidation potential of these enzymes have attracted considerable interest for industrial applications such as biological pulping of paper, fiber bleaching, and remediation of organopollutants such as pesticides, polyaromatic hydrocarbons, PCBs and various halogenated aromatics (including dioxins), certain textile dyes, TNT, and other environmentally detrimental chemicals including cyanides, azide, carbon tetrachloride, and pentachlorophenol.

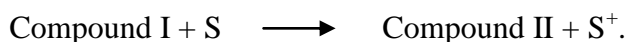
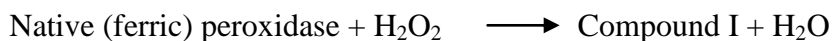
2.4.1 Peroxidase

2.4.1.1 Lignin Peroxidase

Lignin peroxidase (LiP) was first discovered based on the H_2O_2 -dependent C_a-C_β cleavage of lignin model compounds and subsequently shown to catalyze depolymerization of methylated lignin in vitro. Multiple isozymes of LiP are secreted by *P. chrysosporium*, and they have been categorized by their pI and order of elution from a Mono Q anion exchange column. Ten peroxidases are separated by Mono Q chromatography and designated H_1 through H_{10} . Six of these catalyze the prototypical reaction for LiP, the peroxide-dependent oxidization of veratryl alcohol to veratraldehyde. Growth conditions (e.g., N vs. C starved), purification methods, and storage affect relative isozymic levels. Isozymic multiplicity can be explained, at least in part, through dephosphorylation by an extracellular mannose-6-phosphatase.

LiPs are glycoproteins with molecular weights estimated at 38-46 kDa. Enzyme intermediates in the catalytic cycle of lignin peroxidase are analogous to other peroxidases; steady-state and transient-state kinetics have been studied in detail. The interaction of lignin peroxidase with its substrates is by a ping-pong mechanism, i.e., H_2O_2 oxidizes ferric enzyme by two electrons to give compound I

(one oxidizing equivalent as an oxyferryl center and the other in the porphyrin cation radical); compound I oxidizes aromatic substrates by one electron to give compound II (a one-electron oxidized intermediate), which again oxidizes aromatic substrates to return the enzyme to resting state.



Although the assortment of reactions catalyzed by LiP is very complex, the initiation of these reactions is simple. LiP oxidizes the aromatic substrates (indicated as S above) by one electron; the resulting aryl cation radicals (indicated as S^{·+}.) degrade spontaneously via many reactions dependent on the structure of the substrate and on the presence of reactants. Production of cation radical intermediates from methoxybenzenes was conveniently detected by ESR because of the relatively long half-lives of the cation radicals. Using more lignin-related compounds, Hammel *et al.*, (1986) showed the involvement of radical intermediates by identifying radical-dimer products, as well as carbon-centered and peroxy radical intermediates. Lip-catalyzed reactions include C_a-C_β cleavage of the propyl side chains of lignin and lignin models, hydroxylation of benzylic methylene groups, oxidation of benzyl alcohols to the corresponding aldehydes or ketones, phenol oxidation, and even aromatic cleavage of nonphenolic lignin model compounds. Detailed reviews on the radical chemistry of LiP-catalyzed reactions are provided elsewhere.

The secondary metabolite veratryl alcohol is thought to play an important role as a mediator of the oxidations or maintaining an effective catalytic cycle in oxidations of both nonphenolic and phenolic substrates by LiP. The role of the veratryl alcohol cation radical intermediate as a diffusible redox mediator is controversial and an enzyme-bound mediator is argued to be a more likely scenario.

The oxidation of nonphenols by LiP produces phenolics. This explains both the depolymerization of lignin and also the repolymerization of phenolic lignin fragments *in vitro*. Dilute lignin dispersions and low steady-state H₂O₂