PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM *BACILLUS CEREUS* BY USING SAWDUST AS A SUBSTRATE

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JUDUL : **PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM *BACILLUS CEREUS* BY USING SAWDUST AS A SUBSTRATE**

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PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM *BACILLUS CEREUS* BY USING SAWDUST AS A SUBSTRATE

FARIZAH BINTI HAMID

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

Faculty of Chemical & Natural Resources Engineering
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I declare that this thesis entitled "Production of Polyhydroxybutyrate (PHB) from *Bacillus cereus* by using Sawdust as a Substrate" 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."

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Date : 14 April 2010

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 objectives of this research are to determine the production of lignin degradation in sawdust using *Bacillus cereus* and to study the potential of *Bacillus cereus* for PHB production using sawdust as a substrate. There consist of two major parts which are fermentation for delignification of sawdust and production of PHB. For delignification analysis, Klason method is used in order to determine the percentage of lignin content and lignin degrades by using sawdust as a substrate. *Bacillus cereus* act to degrade lignin in the sample. Based on the experiment sample 1 shows the lower percent of lignin content with 38.45% and become the higher percentage of lignin degrade with 13.75%. This is because the bacteria which are *Bacillus cereus* break down the long carbohydrate chains or hemicellulose converted the sawdust into short chains of glucose and eventually individual glucose. While sample 4 shows the lowest percentage of lignin degrade but higher percentage of lignin content. The second part is on the production of PHB. From the result, percentage yield of PHB produced from cellulose and glucose are 1.29% and 3.42%. In conclusion, *Bacillus cereus* has potential to produce PHB using sawdust as a substrate although the yield is smaller. Several precautions must take placed to avoid any mistaken during the experiment. As a recommendation HPLC can be used to obtain more accurate result.

ABSTRAK

Tujuan kajian ini adalah untuk menentukan pengeluaran degradasi lignin serbuk gergaji menggunakan *Bacillus cereus* dan mengkaji potensi *Bacillus cereus* untuk menghasilkan PHB menggunakan serbuk gergaji sebagai substrat. Ada terdiri daripada dua bahagian utama iaitu fermentasi untuk delignifikasi serbuk gergaji dan penghasilan PHB. Untuk analisis delignifikasi, kaedah Klason digunakan untuk menentukan peratusan kadar lignin dan mendegradasi lignin dengan menggunakan serbuk gergaji sebagai substrat. *Bacillus cereus* bertindak untuk mendegradasi lignin dalam sampel. Berdasarkan keputusan percubaan 1 menunjukkan peratus lebih rendah daripada kadar lignin dengan 38,45% dan menjadi peratusan lebih tinggi mendegradasi lignin dengan 13,75%. Ini kerana bakteria *Bacillus cereus* telah memecahkan karbohidrat rantai panjang atau hemiselulosa menukarkan serbuk gergaji rantai pendek menjadi glukosa dan akhirnya menjadi individu glukosa. Sedangkan 4 sampel menunjukkan peratusan terendah mendegradasi lignin tetapi peratusan kadar lignin yang lebih tinggi. Bahagian kedua adalah pada pengeluaran PHB. Dari hasil kajian, peratusan keputusan PHB yang dihasilkan dari selulosa dan glukosa adalah 1,29% dan 3,42%. Sebagai kesimpulan, *Bacillus cereus* mempunyai potensi untuk menghasilkan PHB menggunakan serbuk gergaji sebagai substrat walaupun hasil lebih kecil. Beberapa tindakan pencegahan harus ditempatkan untuk mengelakkan salah selama percubaan. Sebagai cadangan, HPLC boleh digunakan untuk mendapatkan keputusan yang lebih tepat.

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LIST OF SYMBOLS/ABBREVIATIONS

ATP	= adenosine 5'-triphosphate
C	= carbon
Ca^{2+}	= ion calcium
g	= gram
hr	= hour
H_2O_2	= hydrogen peroxide
H_2SO_4	= sulfuric acid
kDa	= kilo Dalton
ml	= mililitre
Mn^{2+} and Mn^{3+}	= ion manganese
N	= nitrogen
OD	= optical density
%	= percent
PHA	= polyhydroxyalkanoates
PHB	= polyhydroxybutyrate
PHV	= Polyhydroxyvalerate
μm	= micrometer
^oC	= degree Celcius

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CHAPTER 1

INTRODUCTION

1.1 Background of study

Today, environment was polluted with waste that not degradable. In Malaysia, 90% of waste placed at landfill which plastics account about 20% by volume of municipal solid wastes and reduce the capacity of precious landfill sites (Lee and Yu, 1997). As it becomes increasingly difficult to obtain new landfill areas due to the awareness of the lay public through the phenomenon called not in my backyard, two alternatives remained namely waste reduction either by recycling or the use of degradable plastics. Plastic is one of the materials are relatively inert and cannot be degraded in natural environment, unlike wood, paper, natural fibres or even metal and glass.

Plastics which thrown into river, ocean and other water made pollution occur. Furthermore, it is danger to environment if plastic is burned because it contains toxic chemical substance. In a survey among the developed countries, in average of 398 kg of domestic waste are generated annually by each person. But now, societal concerns and a growing awareness throughout the world have triggered a product and processes which contributed and loss of environment quality. Most industrialized countries right now have either banned or restrict the use of plastic packaging so as to reduce the environmental degradation resulting from it.

Microorganism that produce and store Polyhydroxyalkanoates (PHA) under nutrient limited conditions may degrade and metabolize it when the limitation is removed (Williams and Peoples, 1996). However, the ability to store PHA does not necessarily guarantee the ability to degrade it in the environment (Gilmore *et al*., 1990). Furthermore PHA is currently limited due to their high costs. Poly-β-hydroxybutyrate (PHB) is an intracellular storage compound, which provides a reserve of carbon and energy in several kinds of microorganism (Khosravi *et al*., 2005) which is very attractive materials in producing bioplastic due to their complete biodegradability.

PHB which is a biodegradable and biocompatible thermoplastic compound has broadly similar physical properties to poly (propylene). It has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture due to its biodegradability (Bucci *et al*., 2005). The amount of plastic waste increases every year and the exact time needed for its biodegradation is unknown. Nowadays plastics and synthetic polymers are mainly produced using petrochemical materials that cannot be decomposed. Therefore they contribute to environmental pollution and are a danger to many animals. During the last decade, much attention has been focused on the production of bacterial polyesters. Different bacterial types of microorganisms produce PHB from renewable sources from sugar and molasses as intracellular storage materials.

In this research microorganisms had been take palced is *Bacillus cereus.* In microbiology, the term bacillus means any rod-shaped microbe (and coccus means a spherical microbe). However, *Bacillus* (written with a capital letter and italicized) refers to a specific genus of bacteria. The family *Bacillaceae* are all Gram-positive, rod-shaped bacteria which form endospores**,** with two main divisions, there are the anaerobic spore-forming bacteria of the genus *Clostridium and* the aerobic or facultatively anaerobic spore forming bacteria of the genus *Bacill Microous.* Characteristically, *Bacillus* cultures are Gram-positive when young, but may become Gram negative as they age.

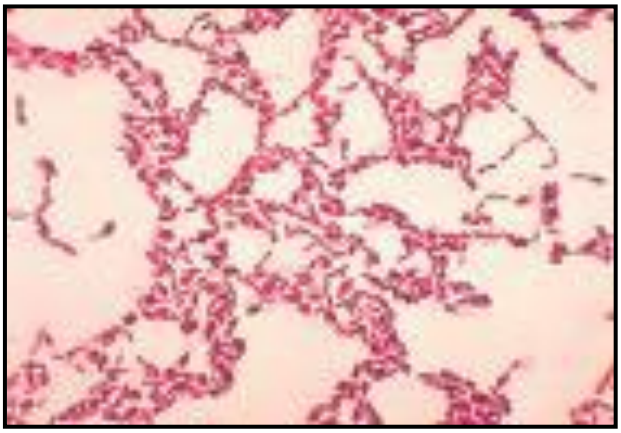

Figure 1.1: *Bacillus cereus*

Bacillus species are aerobic, sporulating, rod-shaped bacteria which are ubiquitous in nature. Gram-stained cells, 1 µm wide, 5-10 µm long, arranged singly or in short chains. *Bacillus cereus* is a spore-forming organism which occurs naturally in most foods. It causes two different forms of food poisoning: an emetic illness and a diarrhoeal illness. The emetic illness is mediated by a highly stable toxin that survives high temperatures and exposure to trypsin, pepsin and pH extremes.

1.2 Objective

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

i. To determine the of lignin degradation of sawdust using *Bacillus cereus.*

ii. To study the potential of *Bacillus cereus* for PHB production using sawdust as a substrate.

1.3 Scope of Study

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

i. To determine amount of the lignin degradation using sawdust by *Bacillus cereus* at optimum temperature (30 ºC), rotation speed (250rpm), volume (250 ml) and pH 7.
ii. To determine the potential of *Bacillus cereus* in producing PHB using sawdust as a substrate at temperature (30 ºC) and rotation speed (250rpm).

1.4 Problem statement

The main purpose of this work is to reduce pollution especially in the ocean. Plastics have shown that these materials are relatively inert and not degradable, unlike wood, paper, natural fibres or even metal and glass. In other instances, plastics which were thrown indiscriminately into the ocean and other water ways have resulted in the suffocation and death of sea animals. Besides, if plastic will release chemical toxic during incineration so it make air pollution that harmful to people. Statement based on EPA United States Environmental Protection Agency state that individual waste generation of 4.5 pounds per person per day which make increasing in landfill.

The amount of plastic waste increases every year and the exact time needed for its biodegradation is unknown. Nowadays plastics and synthetic polymers are mainly produced using petrochemical materials that cannot be decomposed. Therefore they contribute to environmental pollution and are a danger to many animals. During the last decade, much attention has been focused on the production

of bacterial polyesters. Different bacterial types of microorganisms produce PHB from renewable sources from sugar and molasses as intracellular storage materials.

Nowadays global warming is a major concern for many people all aver the world. That is why bioplastics is the subject of a good deal of attention. Bioplastics are the key material which will contribute to the sustainable supply of useful plastics for everyday life without increasing carbon dioxide concentration in the air. By using plastic from PHB, environment will be safe as global warming can be reduced. Plastics and synthetic polymers are mainly produced from petrol chemical elements, which do not decompose, thus resulting in environmental pollution. They are stored, burnt or recycled. During combustion, water and carbon dioxide are released into the atmosphere where increase in the carbon dioxide concentration in the atmosphere occurs. By recycling polymers, the material quality decreases.

Biological polymers are part of a cycle of water and carbon dioxide are used during the photosynthesis in the plant. The bacteria use carbohydrates by fermentation in the manufacture of PHB. In the USA, Europe and Japan it is expected that biodegradable materials will be important due to their material properties being suitable for a wide range of fields. Waste is currently causing serious environmental problems in many countries, especially in industrial countries.

1.5 Rational and Significances

Bioplastics can be save our oil such as petroleum so bioplastic is a alternative way to safe and protect our world from destroy. By using waste like sawdust, vission of ‘waste to wealth’ can be achieved. Production of bioplastic is environment friendly where no need new raw material from environment. In household waste 30 percent are all types of packaging foil such as packaging foils for foods, bags and coated foil on paper. To solve these problems, we should use

environmentally friendly foils (blends) and favors biodegradation on the compost keep instead of burning. Blends can be made and can be used instead like PVC foil.

CHAPTER 2

LITERATURE REVIEW

2.1 Plastic and bioplastic

Plastic is a material made from petroleum capable mould into various shapes. Plastic is the general common term for a wide range of synthetic or semisynthetic organic amorphous solid materials suitable for the manufacture of industrial products (http://en.wikipedia.org/wiki/Plastic,March 2007). By increasing application and production of plastic in industry, environment problem will occur. Electrical and Electronic equipment and Motor Vehicle markets together accounted for 58 percent of plastics demand in 2003 (Medical Device Research. LLC, April 2004). Statistic also found that around 61.5 pounds of plastic per person per year ware produced.

Biodegradable of plastic usually degraded using natural material like vegetable crop derivative or animal such as corn, banana stem waste, potato starch, sugar cane and soy protein. Biodegradable of plastic or bioplastic is the process by which organic substances are broken down by living organisms. Organic material can be degraded and will decompose in the environment aerobically, with oxygen for composition or anaerobically without oxygen for landfill. Plastic are biodegraded aerobically in wild nature, aerobically sediments and landfill and partly aerobically and partly anaerobically in composts and soil. Aerobic microorganism will produced carbon dioxide and water as a final product when oxygen available.

However, under anaerobic condition which is without oxygen, it's responsible to produce microbial biomass, carbon dioxide, methane and water.

Since the 1980's environmental activists have been promoting the concept of degradable plastics along with recycling of trash as the answer to our growing municipal solid waste disposal and litter problems. Reasons for this lack of adoption of these materials include such as lack of physical and thermal properties which meet the end use performance requirements. Beside that cost of the biodegradable polymers (excluding paper) vs. hydrocarbon polymers is lower, processing difficulties with the bioplastics (degradable or non-degradable), degradable bioplastics contaminate the current plastic recycle waste streams and realization that biodegradable plastics do not actually degrade under normal landfill conditions found today in most municipal landfills.

Previously, interests in things such as biodegradable plastics and enzyme efficiency were limited and industrial expertise centre primarily to chemical companies, which was why the scope of innovation was narrow and the time frame was long. Now, interest in industrial biotechnology, also known as white biotechnology, is coming from diverse group of researchers and industries, which will help accelerate the pace that scientific knowledge accumulates and broaden the range of commercial applications for this science. For several decades, plastics derived from fossil fuels have grown at a faster rate than any other group of bulk materials, and expectations are that this high growth trend will continue until 2020. As a more viable and promising alternative, bio-based plastics (bioplastics) or biopolymers, derived from sustainable and renewable resources, could serve to offset, to a certain extent, the non- renewable energy use of the plastics industry. They are usually derived from plant sources such as hemp oil, soy bean oil and corn starch. Bioplastics are biodegradable that can be degraded by microbes under suitable conditions. Some of the important types of bioplastics include Polylactide acid (PLA) and Polyhydroxyalkanoate (PHA).

Malaysia produces more than 60% of resins used for the manufacture of plastics. Malaysia's exports of plastics in its primary forms such as polyethylene, polypropylene, polystyrene, polyvinyl chloride make up 51.5% of plastics exports in 2003. China, Hong Kong, Singapore, Japan and Indonesia are the major consumers of Malaysian plastics (Faizal Ramli, 2008).

The group of biomass for plastics is constantly growing and because of that the recent developments in biochemistry many monomer chemical for plastics will be able to manufactured from biomass resources similar cost to petroleum. The development of polyolefins from bioethanol will significantly increase in the future. JapanBioPlastics Association, Tokyo, Japan state that in 2002, the Japanese government decided on two strategy policies called 'Biotechnology Strategy Guidelines' and 'Biomass Nippon Strategy'. In the 'Biotechnology Strategy Guidelines" the Japanese government set down a clear target for a remarkable increase in the demand for biomass based on plastics.

Microorganism such as bacteria and fungi are involved in the degradation of both natural and synthetic plastic (Gu *et al*., 2000a). These microorganisms convert the bioplastic into carbon dioxide, methane, water and biomass. Certain level of temperature, water, oxygen and food as a source require by active microorganism for effective biodegradation. Biodegradation of plastics can be achieved by enabling microorganisms in the environment to metabolize the molecular structure of plastic films to produce an inert humus-like material that is less harmful to the environment (http://en.wikipedia.org/wiki/Biodegradable_plastic,November 2008).

2.2 Raw material and Carbon Source

To form bioplastic we need material that will produce carbon source which is containing carbohydrate that can be used in biodegradable of plastics such as corn, starch, potato starch, sugar cane, soy protein, palm oil etc. The Freedonia

Group estimates that the demand for bioplastics will increase 20% every year through 2010, with film, bottles and food service products being the largest markets. As an environmental friendly, sawdust had been chosen in this study.

In this research, sawdust is used as a substrate (carbon source). Sawdust is composed of fine particles of wood. This material is produced from cutting with a saw, hence its name. It has a variety of practical uses, including serving as an alternative to clay cat litter, or as a fuel, or for the manufacture of particleboard. Historically, it has been treated as a by-product of manufacturing industries and can easily be understood to be more of a hazard, especially in terms of its flammability. It has also been used in artistic displays, and as scatter. Sawdust is used as a raw material because it easy to found and save. Vision of waste to wealth can be achieved by using sawdust waste because it will safe our world by reduced the landfill space.

Figure 2.1: Sawdust

The search for profitable outlets for sawdust, shavings, and similar wood wastes is often instigated by the need for reducing costs of disposal of materials that clog production, or by the desire to get some return from material that in the log form has represented a considerable outlay of money. In the pelletizing process the raw materials dried, ground and pressed through the holes of a hot die. Important factors in the process are the moisture content of the raw material and the temperature in the densification step. By investigating the formation mechanisms of pellets, (e.g. Rhe´n *et al*., 2005) that raw material moisture content and die

temperature are the main variables for compressive strength and dry density of pellets. Compaction (densification) pressure has a minor influence on these characteristics. Contrary to this, Wild et al. (2007) found that the compaction pressure was an important factor in the process. Both experiments were performed on a laboratory single pellet press but the experimental setup and the compaction pressure ranges in these two trials were not comparable, which might be the reason for the opposing results.

Frequently, installations based on inefficient steam power plants that once used wood waste for fuel at the point of its production have been replaced by oil, gas, or electric equipment, so that many major outlets for sawdust as fuel have been closed. On the other hand, certain uses for sawdust and shavings have been extended. Many uses for sawdust and shavings are open to the individual producers of such waste. Many call for retailing special qualities of material or relatively small lots of material, demands for which are customarily supplied by centralized dealers who specialize in sawdust and shavings.

The composition of the sawdust used for pelletizing has a large impact on the pellet quality. Variability in sawdust age might cause variability in pellet quality. It is generally assumed that a mixture of fresh and stored sawdust improves the durability of pellets and therefore pellet industries use mixed raw materials (Lehtikangas, 2001). Thus stored sawdust increases the friction in the dies during pelletizing resulting in raw material softening, which is necessary for self bonding of wood particles in the pellets and improved pellet durability (Back, 1987; Lehtikangas, 2001). Large amounts of oleophilic compounds inhibit self bonding between individual wood particles in binderless wood composites, like wood pellets, because the active sites for bonding are blocked, eventually resulting in poor compressive strength (Back, 1987).

Monitoring the changes in fatty and resin acid content and the composition of large-scale outdoor stockpiles of sawdust also gives a good measure of the aging process of the sawdust (Arshadi *et al*., 2007). Other factors like changes in moisture content, calorific value, ash content and color changes, which are detected by NIR

spectroscopy (Lestander and Rhe´ n, 2005), have an effect on the maturity of sawdust.

The effect of EB treatment on sawdust for pellet production had a similar effect on the pellet properties as using stored (mature) sawdust. The chemical or physical explanation for this behavior is not yet fully understood. Some changes in the fatty and resin acid composition and in the amount of extrac- tives at different EB doses were found but this did not explain the differences in pellet quality. During storage of sawdust, the amount of fatty and resin acids slowly decreases (Arshadi *et al*., 2007) but this behavior was slightly different for EB treated sawdust. Increased reactivity of the wood components (cellulose, hemicelluloses and lignin) by the formation of free radicals, as a consequence of EB treatment might explain some of the changes in pellet properties but more research is needed to fully understand the effect of EB treatment of sawdust for pellet production.

2.3 Delignification process

Selective delignification is apparent when greater amounts of lignin are degraded relative to the amount of cellulose. Generally cellulose, hemicellulose and lignin substrate were treated to reduce the lignin content by inoculating the substrate containing the substrate with fungus with the temperature in range 15ºC to 40 ºC. In this type of decay, lignin in the secondary wall and middle lamella may be almost entirely removed, whereas large quantities of cellulose in the S2 layer of the cell wall are left intact. Lignin's content in prepared sawdust is (29,16±0,05)%, sample's dryness is (90,32±0,01)%. Residual lignin's content in the treated material was defined using Klason-Komarov method (A.D. Ivahnov *et al*., 2008).

There are two types of hydrogen bonds in cellulose molecules:, those that form between the C_3 OH group and the oxygen in the pyranose ring within the same molecule and those that form between the C_6 OH group of one molecule and the oxygen of the glucosidic bond of another molecule. Ordinarily, the beta-1,4

glycosidic bonds themselves are not too difficult to break. However, because of these hydrogen bonds, cellulose can form very tightly packed crystallites. These crystals are sometimes so tight that neither water nor enzyme can penetrate them and only *exogluconase,* a subgroup of cellulase that attacks the terminal glucosidic bond, is effective in degrading it. The inability of water to penetrate cellulose also explains why crystalline cellulose is insoluble. On the other hand, amorphous cellulose allows the penetration of *endogluconase,* another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. The natural consequence of this difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called *hydrolysis* because a water molecule must be supplied to render each broken bond inactive. Sometimes, wood chips are pretreated with acid at approximately 160°C to strip hemicellulose and lignin before they are treated with an enzyme or a mixture of enzymes. In general, 20 to 70% yield of glucose can be expected after 24 hours.

The conversion of cellulose into glucose is now known to consist of two steps in the enzyme system of *Trichoderma viride*. In the first step, beta-1,4 glucanase breaks the glucosidic linkage to *cellobiose,* which is a glucose dimer with a beta-1,4 bond as opposed to maltose, a counterpart with an alpha-1,4 bond. Subsequently, this beta-1,4 glucosidic linkage is broken by beta-glucosidase:

$$\text{Cellulose} \xrightarrow{\text{b-1,4 glucanase}} \text{Cellobiose} \xrightarrow{\text{b-glucosidase}} \text{Glucose}$$

The kinetics of cellulose hydrolysis has been widely studied, and Michaelis-Menten types of rate expressions with substrate or product inhibition terms have been proposed to describe the observed reaction kinetics (Nam Sun Wang, 1999). In this study, lignin peroxidase is chosen for delignification process. Lignin peroxidase is an enzyme that catalyzes the chemical reaction

1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol + H_2O_2 ⇌3,4-dimethoxybenzaldehyde + 1-(3,4-dimethoxyphenyl)ethane-1,2-diol + H_2O

Thus, the two substrates of this enzyme are 1,2-bis(3,4-dimethoxyphenyl)propane-1,3-diol and H_2O_2, whereas its 3 products are 3,4-dimethoxybenzaldehyde, 1-(3,4-dimethoxyphenyl) ethane-1, 2-diol, and H_2O, (http://en.wikipedia.org/wiki/Lignin_peroxidase, August 2009).

Research by Jiang and Argyropolous state that the elemental analysis from the studied residual lignin samples are exhibited. Nevertheless, the residual lignin from the less delignified pulp sample exhibits a very slight decrease in carbon content and a correspondent increase in oxygen amount, when compared with the residual lignins isolated from the pulps with higher delignification degrees. On the other hand, the residual lignin from the pulp delignified with a higher H factor even if it has the same kappa number, denotes higher carbon content and a corresponding decrease in oxygen content. These features corroborate the existence of more lignin condensation reactions for higher extents of delignification.

The industrial processes of cellulose production have a negative influence on an environment since they used the sulfur that containing reagens for lignin from a wood biomass. A variety of catalysts and catalytic additive for wood delignification process are known (R. R. Hames *et al*., 1998), but only a few of them have found at present the industrial application (T. Vourine *et al*., 1993). Lignin degradation is primarily an aerobic process, and in an anaerobic environment lignin can persist for very long periods (Van Soest, 1994). Because lignin is the most recalcitrant component of the plant cell wall, the higher the proportion of lignin the lower the bioavailability of the substrate. The effect of lignin on the bioavailability of other cell wall components is thought to be largely a physical restriction, with lignin molecules reducing the surface area available to enzymatic penetration and activity (Haug, 1993).

The oxidation wood delignification with acetic acid and hydrogen peroxide is of apparent interest since under this condition of lignin units is observed (S. A. Kuznetsova *et al*., 2000). Cellulose and hemicelluloses are polymers of carbohydrate nature built up from molecules of simple sugar, and lignin is a polymer consisting of phenylpropane units.

2.4 Microorganism

Bacillus cereus is a Gram-positive, facultatively aerobic sporeformer whose cells are large rods and whose spores do not swell the sporangium. These and other characteristics, including biochemical features, are used to differentiate and confirm the presence *B. cereus*, although these characteristics are shared with *B. cereus* var. mycoides, *B. thuringiensis* and *B. anthracis*. Differentiation of these organisms depends upon determination of motility (most *B. cereus* are motile), presence of toxin crystals (*B. thuringiensis*), hemolytic activity (*B. cereus* and others are beta hemolytic whereas *B. anthracis* is usually nonhemolytic), and rhizoid growth which is characteristic of *B. cereus* var. mycoides.

Bacillus cereus is a spore-forming organism which occurs naturally in most foods. It causes two different forms of food poisoning: an emetic illness and a diarrhoeal illness. The emetic illness is mediated by a highly stable toxin that survives high temperatures and exposure to trypsin, pepsin and pH extremes. The diarrhoeal illness is mediated by a heat- and acid-labile enterotoxin. *B. cereus* was found to produce PHB at certain concentration of its dry cell weight, using glucose as the main carbon source (Valappil *et al*., 2007).

2.5 Poly β-hydroxybuyrate (PHB)

2.5.1 Properties and Characteristics

PHB is the only polymer from the group of PHA's to be produced in large quantities. This polymer has poor mechanical properties. They are synthesised by bacteria as storage compounds for energy and carbon, normally in the presence of excess carbon with at least one nutrient essential for growth, such as nitrogen, phosphorus, sulphur or oxygen present in limiting concentration (Anderson and Dawes, 1990). PHB is a partially crystalline material, has good barrier properties such as PVC and PET and can be used in packaging industries as a biodegradable plastic for solving environmental pollution problems.

There are many references to attempts to mix PHB with other polymers with the aim of improving its mechanical properties, unfortunately with only limited success up until now. Most polymers cannot be mixed from a thermodynamic point of view. The chemical incompatibility does not permit a good mixture, i.e., there is no good distribution in other polymers. There are many references to miscible blends containing PHB. PHB is miscible with polysaccharides such as cellulose and starch derivatives, PHB cellulose acetate butyrate, and cellulose acetate propionate.

PHB is a widely distributed intracellular reserve substance typical of prokaryotes. PHB exist in the cytoplasmic fluid in the foam of crystalline granules about 0.5μm in diameter and can be isolated as native granule or by solvent extraction (Anderson, AJ *et al*., 1990). Various researchers have explained that soil bacteria generally produce PHB. Production of PHB will increase if convenient condition is made available. Besides, these biopolymers increase the resistance of bacteria (hanzkova, A *et al*., 1985).

Polyhydroxybutyrate (PHB) is an intracellular storage compound that provides a reserve of carbon and energy in several microorganisms (Anderson AJ *et al*. 1990). Previous research by Universiti Sains Malaysia found that the production of a group of bacterial polyester identified as poly(hydroxyalkanoates) from palm oil, a renewable resource and one of Malaysia primary commodities. The research group has been able to isolate a number of local microorganisms which has been shown to produce a widely used standard plastic for biodegradability namely, poly (3-hydroxybutyrate). The results have so far indicated that palm oil is an efficient

raw material for the production of the plastic as compared to the common raw material, glucose, being used to produce the plastic at the industrial scale.

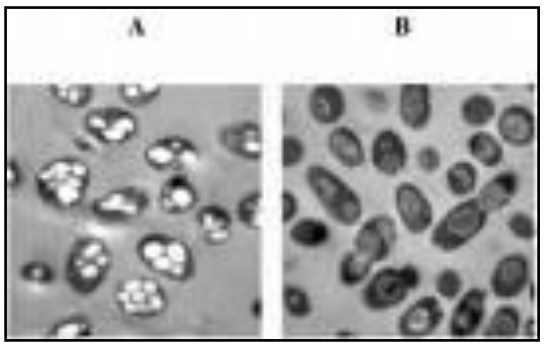

Figure 2.2: Polyhyroxybutyrate (PHB)

2.5.2 Metabolic Pathways of PHB synthesis

The manufacturing process of PHB begins with sunlight. Through photosynthesis carbon dioxide from the atmosphere is converted to carbohydrates via sugar beets or other raw material such as sugar cane and sawdust. These carbohydrates are the raw material for the manufacture of PHB. PHB can be produced from glucose as a raw material or agricultural waste like, for instance, molasses or material, which is refined from the processing of sugar beets and lactose. The sugar splits up in the metabolism to C2 building blocks, which are converted, over several steps, to C4 monomers. Finally, PHB is polymerized. The biosynthesis pathway of PHB is shown in the Figure 2.1.

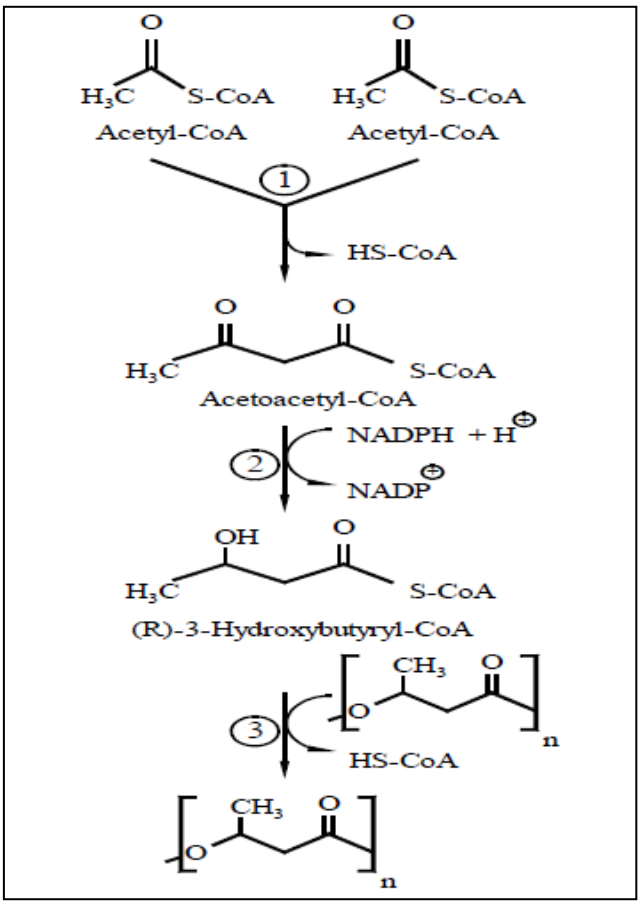

Figure 2.3: Biosynthesis pathway of PHB from acetyl-CoA

2.5.3 Applications

Polyhydroxybutyrate or polyhydroxyvalerate (PHB/PHV) copolymers are used in preference to polyhydroxybutyrate (PHB) homopolymers for general purposes (e.g. moulding containers) in order to obtain a better balance of stiffness and toughness. Polyhydroxyvalerate PHV contents of 5 - 20% give a useful range of properties broadly similar to those of the polyolefins (the polyethylenes and polypropylene). They melt at lower temperatures than the homopolymer, giving a useful improvement in melt-processability. Their other properties are similar to those of polyhydroxybutyrate.

They are being used for biodegradeable containers (of which shampoo bottles are the most high-profile example) and other articles difficult to recycle e.g. disposable razors or medically contaminated articles.

Microorganisms that produce and store PHA under nutrient limited conditions may degrade and metabolize it when the limitation is removed (Williams and Peoples, 1996). For biodegradable of plastic, Poly-β-hydroxybutyrate (PHB) is needed as an intracellular storage material compounds in many microorganisms under unfavorable growth conditions. PHB collectively known as poly(hydroxyalkanoates) (PHA) which is provide and reserve energy source that produce carbon dioxide, methanol and agro-industrial by-product for microorganisms. It has many applications in medicine, veterinary practice, tissue engineering materials, food packaging and agriculture due to its biodegradability (Bucci et al., 2005).

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter will cover all the procedure and process for aerobic fermentation process using *Bacillus cereus.* It will also explain steps of delignification of sawdust and analysis PHB. There is five parts of experiment for this research such as prepare feedstock material, prepare microorganism, fermentation analysis, delignification analysis and PHB analysis. All of these parts are to be discussed in this chapter.

3.2 Feedstock Material

Sawdust as a raw material was dried in tray dryer at 45°C for three days. Sawdust then was stored in a cool and dry.

3.3 Microorganism Preparation

For this research *Bacillus cereus* is used in fermentation process.

3.3.1 Agar preparation

For agar preparation, 10 g of peptone, 2 g of yeast extract, 1 g of $MgSO_4.7H_2O$ and 15 g of agar powder are prepared. All of them are put in 1000 ml Schott bottle and distilled water is added until 1000 ml for preparation of 1000 ml agar solution. After that, the Schott bottle is heated and stir by using magnetic stirrer until the mixture mixed well. Next, the solution needs to sterilize in autoclave around 20 minutes at 121^0C. Then, the agar solution is poured into Petri plates and sealed it. The agar solutions were left in room temperature until it hard and cool. Lastly all of the solution was placed in refrigerator at 4^0C and can be used anytime.

3.3.2 Subculture bacteria (*Bacillus cereus*)

Pure *Bacillus cereus* in the tube is taken for culture bacteria process. Firstly, the loop include the wire is heated using Bunsen burner until it become bright red to make sure it is free from foreign microorganism. Secondly, the cap is removed and flame the neck of the tube without place the cap on the table. 1 loop of *Bacillus cereus* is taken from the tube then streak on the agar surface by using streak plate procedure. Before return the cap to the tube, flame the mouth of the tube and the cap again. Next, the agar is left overnight at 30^0C. This procedure is done near the Bunsen burner in laminar flow. The laminar flow is wiped away with ethanol before and after cultivation of bacteria process.

Figure 3.1: *Bacillus cereus* on agar surface

3.3.3 Broth preparation

A 1000 ml broth solution is prepared in 1000 ml Schott bottle. Broth nutrient containing 10 g of peptone, 2 g of yeast extract, and 1 g of $MgSO_4.7H_2O$ are poured into Schott bottle. After that, distilled water is added until 1000ml. Then, the solution is heating and shaking until it mixed well. Before sterile the solution, loose the Schott bottle cap and cover with aluminum foil. Stick the sterile sticker as a sign that the Schott bottle has been sterile when black line is shown after finish sterilization process. The broth solution then was sterilized in autoclave at 121^oC for 20 minutes.

3.3.4 Inoculums preparation

The nutrient consist of 10 g of peptone, 2 g of yeast extract, and 1 g of $MgSO_4.7H_2O$ were prepared by distilled water until 1000ml. Inoculums preparation is same procedure like broth preparation. 250ml is poured in conical flask then sterile the solution at 121^oC for 20 minutes. In laminar flow, loop was flame using Bunsen burner until it becomes bright red. Next, bacteria are taken one blotch from agar plate (after cultivation one day) then transfer into the solution. The loop was

flamed again and the conical flask that containing solution and bacteria then was put in shaker incubator for three days at 30°C, 250rpm. Next to make the microorganism active, 10ml of inoculums or liquid suspension was transfer into 90ml of broth solution and leaved in shaker incubator for 30 hours at 30°C and 250rpm. For activation process, the solution then was incubated for another 18 hours at the same parameter.

3.4 Fermentation Analysis

10 g of sawdust from feedstock material is weighted and 225 ml of broth medium were put in four round conical flasks. All of the conical flacks were sterilized in autoclave. 25 ml of liquid suspension of *Bacillus cereus* from inoculums preparation were added onto three conical flasks that contain sawdust and broth medium. While another one conical flask had been prepared without inoculums as a control. All of the round conical flasks include uninoculated are incubated aerobically at 30°C in shaker incubator at 250rpm and harvested for 48 hours. After that, the entire samples are filtered using filter paper. Next, the filter papers contain biomass and bacteria are heated in oven at 80°C for three hours. Lastly, weight again the sample and used Klason method for delignification process.

Equation to calculate percentage of lignin content in the sample:

$$\%\ of\ lignin\ content\ in\ the\ sample = \frac{final}{initial} \times 100\%$$

(Equation 3.1)

3.5 Delignification Analysis

For delignification analysis, Klason method is used to calculate the percentage of lignin sample and percentage of lignin degradation. 1 g of sample that had been filtered from fermentation analysis was weight and put in 10ml beaker. Based on Klason method, 20 ml of 72% sulfuric acid (H_2SO_4) was added to the sample and leave at room temperature for two hours. Next, 500 ml of distilled water was added to the sample then heated at 100 °C, 70 rpm in shaker water bath for two hours. The sample then was filtered by using vacuum filtration and washed with distilled to make sure the sample is free from acid. Check the pH of the sample by using litmus paper. Since the sample is neutral, the entire sample has put in the oven to make it dry at 60 °C for one hour. Then the sample was places leave in desiccators to make sure the sample is free from moisture for two days. Finally, the samples were weight and measure the percentage of lignin degradation.

Equation to calculate percentages of lignin degraded in sample:

$$\%\ lignin\ degraded = \frac{\%\ lignin\ control - \%\ lignin\ sample}{\%\ lignin\ control} \times 100\%$$

(Equation 3.2)

3.6 Production of PHB (PHB analysis)

Determination of the amount of PHB was performed chemically. Bacteria were grown on nutrient broth at 30°C for 48 hours on a shaker same like fermentation process. 10 ml of suspensions of cultures were centrifuged at 4 ^{0}C and 5000 rpm for 12 minutes. Then the pellets were suspended with 10 ml of 0.625% sodium chloride, NaOH. After that, the suspended was centrifuge again at the same parameter (4 ^{0}C and 5000 rpm for 12 minutes). Remove the supernatant and pallet

was resuspended again by adding 10ml of 100µm hydrogen peroxide, H_2O_2. The mixture was heated in water bath at 30 ^{0}C for 4 hours. Next the tubes were centrifuged at 4 ^{0}C and 5000 rpm for 12 minutes. To obtain precipitate, 10 ml of chloroform was added, and the tubes were transfer into a glass of Petri dish. 5 ml of concentrated hydrochloric acid, HCl was added and the mixture was heated at 100 °C in a water bath for 10 minutes. After cooling to 25 °C (room temperature), the amount of PHB was determined spectrophotometer or UV-Viss at 235 nm. Repeat the same method by using glucose as a substrate and determine the amount of PHB also using spectrophotometer at 235 nm.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Introduction

In this project by title production of PHB from *Bacillus cereus* by using sawdust as a substrate will divide by four major parts. The first part is growing of *Bacillus cereus* in order to acquaint the lifetime of *Bacillus cereus* at optimum condition. In this part fermentation analysis could be done and the process is carried out in aerobic condition. The second part is delignification analysis to produce cellulose. The process then was proceeding to production of PHB by using the cellulose (from sawdust delignification process) as a substrate.

4.2 Growth curve of *Bacillus cereus*

Before fermentation analysis is done, growth curve of *Bacillus cereus* must be done to figure out the lifetime of the microorganism. The bacteria from agar have transfer into medium using inoculating loop. It then needs to growth by incubate in liquid suspension for 30ºC and 250 rpm for 30 hours. After around of hour, the color of liquid suspension was change from light yellow to cloudy. This is happened because the bacteria have cultured increasing by time. The process then was proceed for another 18 hours for activation process at the same parameter. Table 4.1 below shows the result gained from the experiment and figure depicts the graph of OD versus time that had been plotted as a growth curve of *Bacillus cereus.*

Table 4.1: Data of OD over time of *Bacillus cereus* and blank

Hours	Time	Optical density,abs	
		Blank	***Bacillus cereus***
3	11.30am	0.000	1.475
6	2.30pm	0.000	1.704
9	5.30pm	0.000	2.523
12	8.30pm	0.000	2.125
15	11.30pm	0.000	2.409
18	2.30am	0.000	2.699
21	5.30am	0.000	2.409
24	8.30am	0.000	2.671
27	11.30am	0.000	2.568
30	2.30pm	0.000	2.579
36	8.30pm	0.000	2.584
42	2.30am	0.000	2.638
48	8.30am	0.000	2.745
54	2.30pm	0.000	2.740
60	8.30pm	0.000	2.342

From the table above, optical density of *Bacillus Cereus* increase by increasing of fermentation time. This is happen because the microorganism life and increasing bred at optimal condition (30ºC and 250rpm). This result is logic as theoretically *Bacillus Cereus* growth at optimum temperature of 30-37ºC with minimum pH for growth is 4.3 and maximum pH around 9.3 (Granum PE et al., 1997). The table also shown the maximum reading of OD is 2.745 abs at 48 hours. The readings of OD then decrease by time because the microorganism is unactive or going die.

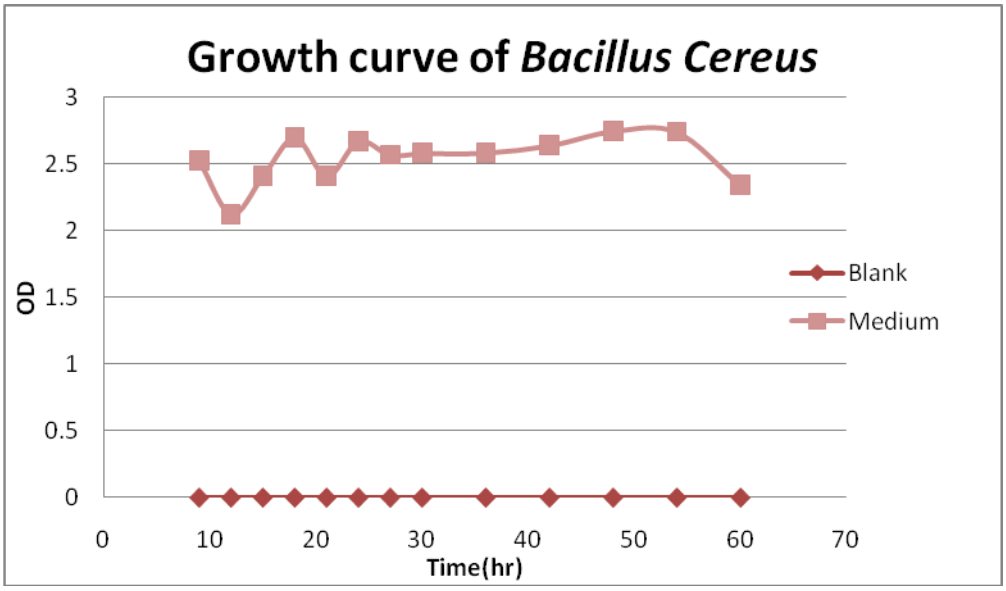

Figure 4.1: Graph of growth curve of *Bacillus cereus*

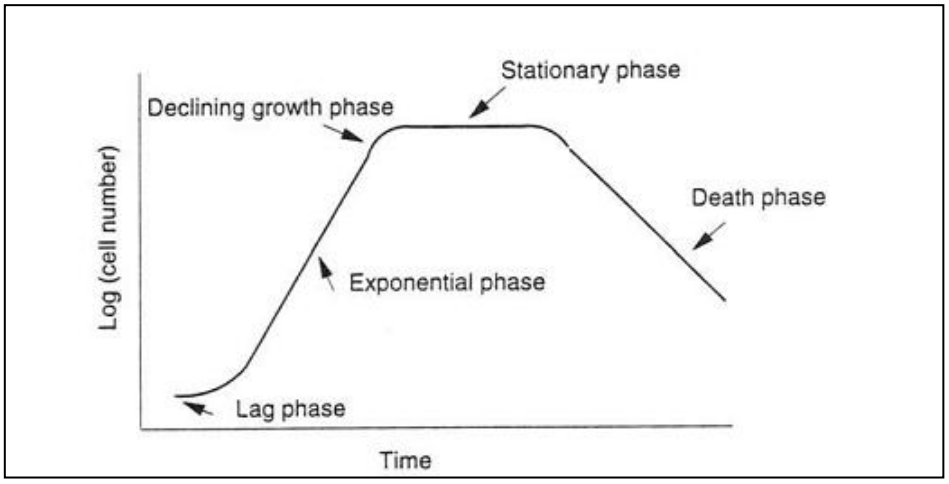

Figure 4.2: Graph of Standard Growth Phase in Batch Fermentation Process.

The both graph shows the growth curve of *Bacillus cereus* and standard growth phase in batch fermentation process. The graph depicts five major phases of bacteria lifetime which are lag phase, exponential phase, declining growth phase, stationary phase and death phase. Based on Figure 4.1, lag phase not occur at the starting time followed by exponential phase where occur at two to 29 hours where *Bacillus cereus* start bred. In this process, the *Bacillus cereus* is given time to adapt with the new environment after become inactive for a long period time. Therefore, when running the fermentation process, the *Bacillus cereus* is already adapting into

the new environment and becoming active. Besides, it may cause for many reasons. For example, when the cells are placed in fresh medium, they might have to adapt to it or adjust the medium before they can begin to use it for growth. Another reason for the lag phase might be that the inoculum is composed partly of dead or inactive cells. If a medium consists of several carbon sources, several lag phases might appear. This phenomenon is called diauxic growth. Microorganisms usually use just one substrate at a time and a new lag phase really results when the cells adapt to use the new substrate.

At 30 to 40 hours it in stationary phase until achieved 48 hours it become more active. When a substrate begins to limit the growth rate the phase of the declining growth begins. These phases occur at 30 to 40 hours where the growth rate slows down until it reaches zero and the stationary phase begins. In the stationary phase the number of the cells remains practically constant, but the phase is important because many products are only produced during it. This may result from a balance between cell division and cell death or the population may simply cease to divide though remaining metabolically active. Microbial populations enter the stationary phase for several reasons. One of the factors is nutrient limitation. At hour 60, the bacteria bred will stop in the death phase. As a conclusion, the lifetime of *Bacillus cereus* is 48 hours at 30ºC and 250rpm.

4.3 Delignification Analysis

After finished the process of growth curve of *Bacillus cereus* and fermentation process, proceed to delignification analysis. Delignification analysis is used to break hemicelluloses from sawdust to get cellulose as a carbon source for production of PHB. Cellulose, the major component of the lignocellulosic wastes from sawdust can be converted into glucose suitable for further microbial processing through its total or partial hydrolysis. Partial cellulose hydrolysates may well be used as rich in energy fodder or as an excellent medium for obtaining single cell protein. The chemical compound surrounding the cellulose in plants like lignin also

limit the diffusion of the enzyme into the reaction site and play an important role in determine rate of hydrolysis. Enzyme hydrolysis breaks down the long carbohydrate chains making up sawdust into short chain of glucose (a simple 6-carbon sugar) and eventually to individual glucose.

In this part, Klason method is used for delignification analysis which consists of several parts. Starting with drying of sawdust at 45ºC to remove moisture and make sure the weight is same and constant. 1 g of dried sawdust was immersed in 20 ml of sulfuric acid and leave at room temperature about 25ºC for two hours. Sulfuric acid act to dissolved all polysaccharide compounds in sawdust sample. After that, the sample transfer into shake flask and diluted with 500 ml of distilled water to ensure that the sample is fully dissolved. The shake flask is left in shaker water bath at 100ºC and 70 rpm for 2 hours. The sample then needs to filtered and washed with distilled water in order to remove the remaining acid. Dried in oven and then put in desiccators to remove moisture content. The percent of lignin content and lignin degrade have calculated. Table 4.2 shows the result.

Table 4.2: Percent lignin content and percent lignin degrade

Sample number	Dry weight, g		Lignin content, %	Lignin degrade, %
	Initial	Final		
1	1.0014	0.385	38.45	13.75
2	1.0000	0.399	39.90	10.50
3	1.0034	0.3998	39.84	10.63
4 (uninoculated)	1.0024	0.4469	44.58	-

Based on the observation from Table 4.2, the percent lignin degrade are 13.75%, 10.50%, and 10.63%. From the result, sample 1 is the highest percent lignin degrade with 13.75% and the lowest percent lignin content. This is because the bacteria which are *Bacillus cereus* already break down the long carbohydrate chains or hemicellulose making up sawdust into short chains of glucose and eventually individual glucose. While sample 4 shows the lowest percentage of

lignin degrade but higher percentage of lignin content. This is because no *Bacillus cereus* in the sample to break down the long chain of the sample. So from the result, the objective is achieved where Bacillus cereus is able and potential to degrade sawdust.

4.4 Production of PHB

PHB production contributes several major parts. The method will start with centrifuge 10 ml of sample at 5000 rpm and 4ºC for 12 minutes. The pallet form from centrifuge was resuspended with 10 ml of sodium chloride and the mixture was centrifuge again. These process also known as treatment process. The pallet is resespended again with hydrogen peroxide. The purpose is to break down the cell wall. Higher cell wall permeability and exposed PHB to the next step (addition of chloroform). Hydrogen peroxide will affect PHB and act to prevent excessive destruction of PHB phase and also call as a lysis agent. After that the solution is leaved in shaker water bath at 30ºC for four hours to make sure the solution of mixture are completely dilute. The mixture then is centrifuge for the last time and the pallet was resespended with chloroform for extraction method. Chloroform is used in order to recover PHB with less polymer degradation. Next, the samples were dried in Petri dish and add 5 ml of hydrochloric acid to convert crotonic acid. Crotonic acid is an insoluble unsaturated carboxylic acid produce by oxidation. Then, transfer the samples to test tube and boil in water bath at 30ºC around 30 minutes. The amount of PHB is determined using spectrophotometrically at 235 nm (Bonartseva and Myschkina, 1985; Kuniko et al., 1988). Table 4.4 shows the data obtained from the experiment.

Name of sample	Optical density	Concentration (mg/ml)	Dry cell weight (mg)	PHB Yield (%)
Sample 1 (cellulose)	1.0597	72.0	9.3	1.29
Sample 2 (glucose)	0.362	22.5	7.7	3.42

Table 4.3: Percent yield of PHB produce by using sawdust as a substrate

Based on Table 4.4, the yield percentage of PHB produced by cellulose is lower than glucose with 1.29%. This is happened because of several factors. Sawdust will produce PHB but at a lower percentage because the amount of cellulose in sawdust is lower compare to glucose. At optimal nutrient for growth of *Bacillus cereus*, it can produce PHB. Nutrient limitation is necessary to trigger PHB accumulation. May be during experiment the reaction containts of nitrogen gas because theoritically at low nitrogen concentrations, PHB content is increasing. So PHB production will decrease by increasing the amount of nitrogen gas. Another factor that made the lower percentage of PHB is leave the sample at a long time and cover the test tube with aluminium foil. By leave it at long time, the hydrogen gas will release and react with the aluminium foil so it may be a reason the PHB prouction is low. The reaction occur by addition of hydrochloric acid is shown below:

PHB + HCL $\longrightarrow$ Crotonic acid

$$-\{O-CH(CH_3)-CH_2-(C=O)\}_n + 2HCL \longrightarrow CH_3CH{:}CHCOOH + Cl_2 + H_2$$

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this research, the aim objective are to determined the production of lignin degrade in sawdust using *Bacillus cereus* and to study the potential of *Bacillus cereus* for PHB production using sawdust as a substrate. According to Valappil *et al. B. cereus* was found to produce PHB at certain concentration of its dry cell weight, using glucose as the main carbon source. Based on the research, *Bacillus cereus* was able to degrade lignin about an average percentage 11.63% at 30^0C by using sawdust as a substrate. In this research, Klason method is used to determine the percent lignin content and percent lignin degrade same method by referring A.D. Ivahnov *et al.* used to defined residual lignin's content in the treated material in 2008. So as a conclusion, *Bacillus cereus* has potential to produce PHB using sawdust as a substrate according to Valappil et al. statement, *Bacillus cereus* was found to produce PHB at certain concentration of its dry cell weight, using glucose as the main carbon source.

5.2 Recommendation

Recommendation is needed in order to improve the experiment since the data gain not very accurate. There are several ways to improve the research such as by using Whatman glass microfiber filters during filtration are the best option so far considered, since Whatman ashless filters and Whatman qualitative filters were burnt when they were put in the oven at 80^{o}C after being used to filter the samples. Besides, for analysis, we will obtain more accurate result if we use (High Performance Liquid Chromatography) HPLC compare to spectrophotometer because HPLC is more sensitive to detect PHB form in the solution. Another way is use anaerobic condition because low condition of oxygen will increase the production of PHB because PHB need more carbon sources for production of PHB.

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APPENDIX A

CALCULATION FOR YIELD PERCENTAGE OF PHB PRODUCTION

A.1 For cellulose (from sawdust)

After centrifuge with 10 ml of sample:

Mass substrate = (72mg/ml)(10 ml)

= 720mg

% $Y_{x/s}$ = (9.3mg)/(720mg) x 100%

= **1.29%**

A.2 For glucose

After centrifuge with 10 ml of sample:

Mass substrate = (22.5mg/ml)(10 ml)

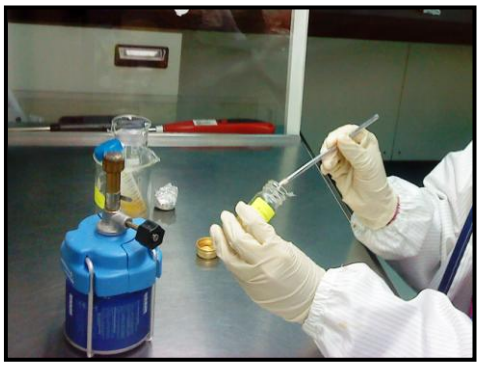
= 225mg

% $Y_{x/s}$ = (7.7mg)/(225mg) x 100%

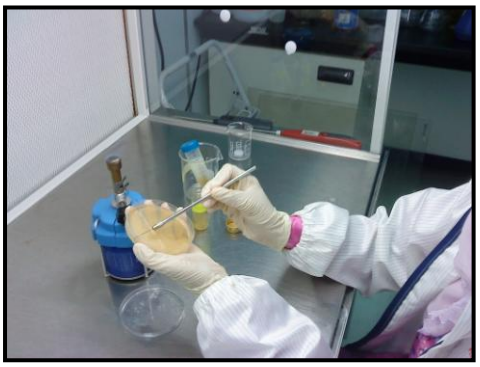
= **3.42%**

APPENDIX B

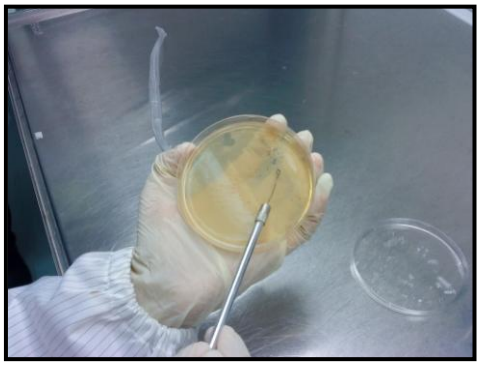
REVIVAL OF *Bacillus cereus*



Bacteria were taken from tube



Transfer bacteria in agar plate

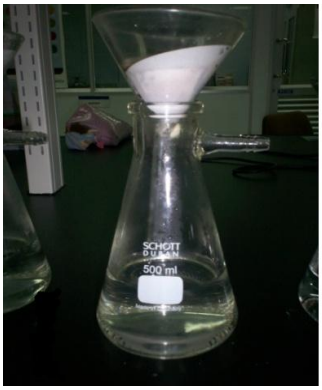


Streak bacteria on agar surface

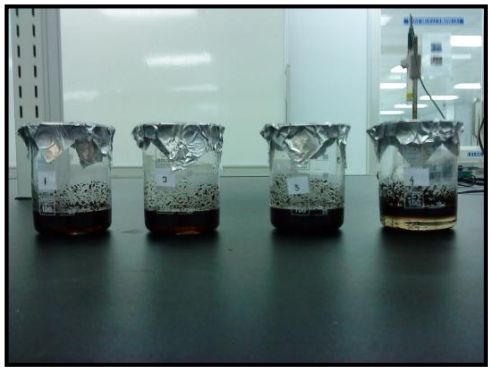
Bacillus cereus after 24 hours

APPENDIX C

ANALYSIS FOR DELIGNIFICATION METHOD

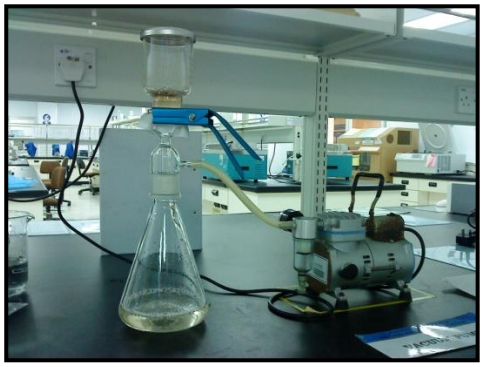


Step 1: Sawdust was filter and dried in oven



Step 2: Sample was added with H_2SO_4

Step 3: Dilute with 500 ml H_2O and put in hot water bath



Step 4: Filter and wash with distilled water

Step 5: Check pH then dry in oven

Step 6: Put in desiccators

APPENDIX D

DATA AND GRAPH FOR STANDARD PHB

Table D 1: Data for standard PHB

Concentration (mg/ml)	Optical density
0	0
20	0.292
30	0.423
40	0.686
50	0.765
60	0.879
70	1.064
80	1.218
90	1.304
100	1.52

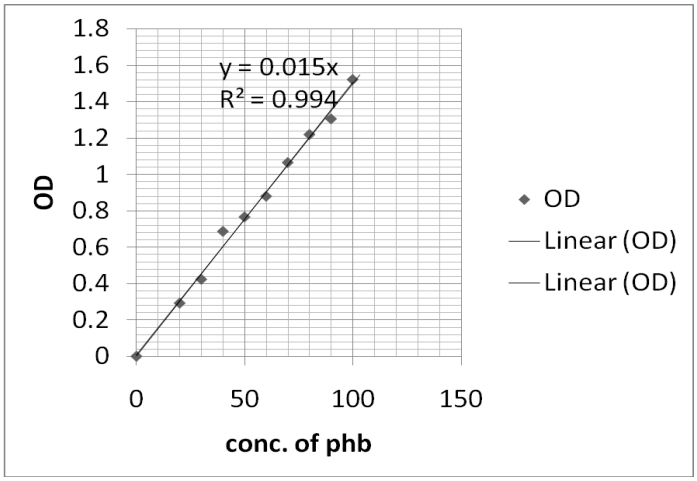


Figure D 1: Graph of Optical density versus concentration of PHB