PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM *Bacillus cereus* BY USING RICE STRAW AS SUBSTRATE

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Date : 13 April 2010

PRODUCTION OF POLYHYDROXYBUTYRATE (PHB) FROM *Bacillus cereus* BY USING RICE STRAW AS SUBSTRATE

NOR SYAFARAH BINTI ZAKARIYA

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
Universiti Malaysia Pahang

I declare that this thesis entitled "Production of polyhydroxybutyrate (phb) from *Bacillus cereus* by using rice straw as 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 : 13 April 2010

A dedication from heaven to earth
specially to my parents,
my families,
my friends and staffs.

None shall compare to
your love and support.

Thank you.

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ABSTRACT

In this paper, there were 2 main objectives. Those were to To determine the potential of delignification rice straw by using *B.cereus* and to study the PHB production from *B.cereus* by using rice straw as substrate. Delignification was crutial due to its chemical durability makes it indigestible to organisms because of its bonding to cellulose and protein material. This lignin sheet acts as a barrier towards the outside elements (Carraher, 2008). The method used for delignification process was fermentation of *B.cereus* with 250 mL distilled water, 0.5 g yeast extract, 2.5 g peptone, 0.25 g $MgSO_4.7H_20$ and 10 g of rice straw in a 500 mL shake flask. Then, the bacteria were fermented in the medium at 30 ºC and 250 rpm. For the analysis of delignification of rice straw by *B.cereus*, Klason's method was utilized. From the result obtained, the highest lignin content was 4.56 % and the Control was 8.18 %. The percentage of the highest lignin degradation for the three samples was 98 %. The average lignin degradation was 78.67 %. After delignification, the bacteria could reacted on the cellulose content to synthesis PHB. The bacteria was fermented in 250 mL distilled water, 0.5 g yeast extract, 2.5 g peptone, 0.25 g $MgSO_4.7H_20$ and 1 g of delignified rice straw in a 500 mL shake flask. Then, the bacteria was fermented in the medium at 30 ºC and 250 rpm. The method of analysis of PHB yield was by using UV-vi spectrophotometer at 238nm. From the results obtained, the highest PHB yield is produced in Medium B; the medium consisted of $MgSO_4.7H_2O$ and yeast that yield 2.62 %. By these results, the objectives were achieved.

ABSTRAK

Terdapat dua objektif utama kajian ini iaitu untuk degradasi lignin dari jerami padi dan untuk menghasilkan PHB kedua-duanya daripada *B.cereus*. Pendeligninan penting kerana struktur kimianya yang menghubungkan selulosa dengan protein serta kompleks menyebabkannya sukar untuk dicernakan oleh organisma. Lignin bertindak sebagai penghalang terhadap element luar (Carraher, 2008). Proses yang digunakan untuk degradasi lignin ialah dengan menapaikan bakteria ke dalam media yang mengandungi 250 mL air suling, 0.5 g ekstrak yis, 2.5 g pepton, 0.25 g $MgSO_4.7H_20$ dan 10 g jerami padi di dalam 500 mL kelalang kon. Kemudian, bakteria dibiarkan dalam keadaan 30 ºC dan 250 rpm. Untuk menganalisa degradasi lignin, 'Klason's method' digunapakai. Berdasarkan kajian, peratus lignin tertinggi ialah 4.56 % dan Kawalan ialah 8.18 %. Manakala peratus degradasi lignin tertinggi 98 %. Manakala, purata delignifikasi ialah 78.67 %. Peratus delignifikasi ini menunjukkan nilai lignin yang berjaya dicernakan oleh bakteria. Selepas itu, proses seterusnya adalah pencernaan selulosa untuk menghasilkan PHB. Proses yang digunakan ialah dengan menapaikan bakteria ke dalam media yang mengandungi 250 mL air suling, 0.5 g ekstrak yis, 2.5 g pepton, 0.25 g $MgSO_4.7H_20$ dan 1 g jerami padi yang telah didegradasi lignin di dalam 500 mL kelalang kon. Untuk menganalisa penghasilan PHB, 'UV-vis spectrophotometer' digunakan pada 238 nm. Daripada hasil kajian, penghasilan PHB tertinggi ialah 2.62 % di dalam 'Medium B' iaitu media yang mengandungi $MgSO_4.7H_2O$ dan yis.

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ATP	= adenosine 5'-triphosphate
C	= carbon
Ca^{2+}	= calcium ion
COASH	= coenzyme A
Fe^{3+}	= ferum ion
g	= gram
hr	= hour
H_2O_2	= hydrogen peroxide
K^+	= potassium ion
kDa	= kilo Dalton
L	= liter
$MgSO_4.7H_2O$	= magnesium sulphate (hydrated)
mL	= mililiter
Mn^{2+} and Mn^{3+}	= manganese ion
MT	= metric ton
N	= nitrogen
NaCl	= sodium chloride
NAD^+	= nicotinamide adenine dinucleotide
NADPH	= nicotinamide adenine dinucleotide phosphate
nm	= nanometer
OD	= optical density
R	= carbon chain
rpm	= rotational per minute
S^+	= sulphur ion
µm	= micrometer
^oC	= degree Celcius

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The field of study for production of polyhydroxybutyrate (PHB) is revolve around polymer. Polymers are large molecules made up of repeating units of smaller molecules called monomers (Smith, 2006). Others included in the category polymers are important compounds such as proteins and carbohydrates. They also include such industrially important plastics such as polyethylene, poly(vinyl chloride) (PVC) and polystrene (Smith, 2006). Polymers that are biodegradable and made up entirely from biological products are called biopolymers. Polyhydroxybutyrate is included as biopolymer. In research filed of biopolymers, it has growing popularity among scientist as well as engineers each and every day (Gross & Kalra, 2002). Kennedy and Sundquist (1993) also summarize the general introduction of biopolymers, technical overview of biopolymer field and description regarding researches on biopolymer in Europe, Japan and Unites States of America. In addition, Lenz (1995) has made a packed summarize on 27 polymer researches that has been conducted in Japan. These shows that biopolymer is a significant compound as an alternative towards contemporary plastic today, that is unbiodegradable.

As mentioned above, biodegradable biopolymer (BDP) is an alternative for the petroleum derived plastic (such as polyethylene). Some BDP can degrade within days or months. The biodegradability is determine by the molecular structure (M Flieger *et al.,* 2003). PHB has a unique molecular structure. Eventhough it has the

same characteristic as polyethylene (but it is brittle), it is biodegradable as it is derived from biological compound such as bacteria or fungi.

Many researches has been done to obtain PHB due to its characteristic as a thermoplastic. Many of the researches to obtain PHB has been conducted by using bacteria. One of the it has been done on *Pseudomonas* sp. that was isolated from Antarctic environment with high stress resistant (D. Ayub *et al.,* 2004). Bacteria has a system to survive in a nutrient starvation condition and tolerate exposure to multiple stress agents. While running the research, the scientists were screening the PHB producing strains in *Bacillus* genus within the antarctic samples by using the classic heating method. But, what they have found is *Pseudomonas* sp. 14-3 strain that can produce high yield of PHB. The environment in Antarctica is always pressurised organisms there. Thus *Pseudomonas* sp. can produce high yield of PHB. Thus, this research contributes to the world of BDP greatly.

Besides *Pseudomonas* sp., other bacterias that has been used are those in activated sludge. A research has been done on synthesis of PHB from activated sludge by W.F.Hu *et al.* (1997). In the activated sludge, bacteria that can be isolated are *Alcaligenes* spp., *Pseudomonas* spp., recombinant *Escherichia coli* and a number of filamentous genera. This mixed culture were able to accumulate polyhydroxyalkanoates (PHA) and their copolymers as secondary metabolite or intracellular carbon reserve when unfavourable environmental conditions are encountered. This research contributes by proving that bacteria that is easily to be obtained can produce BDP and it can lower the cost of production of it. Another research is optimization of PHB production by *Bacillus* sp. CFR 256 strain with corn steep liquor (CSL) as its nitrogen source (S. V. N. Vijayendra *et al.,* 2007). The objective of the study was to economize the PHB production by optimizing the fermentation medium using steep liquor (by-product of starch processing industry) as a cheap nitrogen source by *Bacillus* sp. CFR 256. From the results obtained, the maximum PHB yields were found at highest CSL concentration (25 g/L). Even by using the cheap CSL, yield of PHB can be optimized. This research is important to reduce the production cost of BDP.

A state of the art technology is currently being research. Azuyuki Shimizu (2002) was doing a review on metabolic pathway analysis with emphasis on isotope labeling approach. The objective of this research is to treat metabolism as a network or entirely instead of individual reactions. The bacteria applied for PHB production are *Ralstonia eutropha* and recombinant *Escherichia coli*. At the end of the research, investigation on metabolic flux analysis with gene and protein expressions to uncover the metabolic regulation in relation to genetic modification or change in the culture condition. The research contributes in determine the optimum metabolic pathway to produce PHB and by genetically modify the bacteria, only PHB pathways will be triggered thus no or negligible by-product will be produced.

BDP is superior not by just on its own, but it is highly functional when mixed together with other substance. A very novel study that could be importance for the future is that of functionalized cellulose nanofibers and nanocrystals blended with biodegradable polymers and arclylic acid polymers. This is due to the nanocrystals were found to be markedly superior reinforcing agents than wood flood flour. Their behaviour is also similar to the exfoliated clays in terms of reinforcing properties (Varma, 2005).

1.2 Objectives

The objectives of this study are:

1. To determine the potential of delignification rice straw by using *B.cereus*
2. To study the PHB production from *B.cereus* by using rice straw as substrate

1.3 Scopes of Study

By using the objectives as basis, the scope of study are as follows:

1. Delignification of rice straw by *B.cereus* at optimum condition of pH 7, 30 ºC and 250 rpm.
2. PHB production by *B.cereus* at optimum condition of pH 7, 30 ºC and 250 rpm while the variables are the medium content of yeast, peptone and $MgSO_4.7H_2O$.

1.4 Problem Statement

Within the last few decades, plastic has revolutionized our lifes. Nowadays, plastics are everywhere, almost a must in everyday life. Most plastics are made of polyethylene chain of monomer, methylene (CH_2). The chain is commonly as long as from 1000 to 10000 of repeated CH_2. Due its long chain, hence it is spoken of as high polymers or macromolecules (J. Brydson, 1999). Its molecular structure contributes to unbiodegradability of it. In soil, plastic will takes up to 1000 years to degrade. Whereas in water, it will take 450 years to degrade. 260 million tonnes of plastic are used globally annually, accounting approximately 8 % of world oil production (Thompson *et al.,* 2009). Eventhough plastic usage are inexhaustible, most of it will be disposed off within a year or so after the manufacture (Barnes *et al.,* 2009). This will increase the solid waste from plastic.

Due to its nonbiodegradable property, it can endangered the wild life, marine life especially. Plastics in the ocean are called marine debris. Within 20 years since 1971, the total fish caught by the fishermen reduced by 90 %. The most obvious dwindled is the deep ocean species (Journal Fisheries Research, 2006). It is assumed that pelata fish will be instinct by the observations that showed that on 2005 and 2006, 1 tonne of the fish is caught. But, on 2000, the fish caught was 1621 tonne (Malaysian Inshore Fishermen Action Network, 2009). Marine debris ahave affected at least 267 species world wide, this includes 86 % of all sea turtle species, 44 % of

all sea bird species and 43 % of marine mammal species (Laist, 2997). Those are affected by ingestion, starvation, suffocation and entanglement. In 1980s, researches estimated that 100000 marine mammal deaths annually in the North Pacific related to entanglement in plastic nets and fishing line (Wallace, 1985). These proves that the plastic is affecting the marine lives. The plastic are looking edible thus they consume it. Polyethylene are not biodegradable, thus it will stay in their system. The toxicity in the polyethylene will consume the marine lives slowly and soon, they will die.

Apart from plastic, rice straw is also a liability. From 100 % of rice straw avalaibility after the harvesting season, 51 % from rice straw is thrown away by burning. This causes pollution. So, liability can be converted to assets. These assets are PHB that can contribute to two benefits; reduce air pollution by utilise the rice straw and produce BDP from PHB. Also, potential of availability of rice straw is very high because of the plantation of the rice will yield 0.45: 0.55 for rice straw with rice itself. This ratio were calculated by assuming that every hectare of paddy field will produce 4 to 5 tone of rice and that mean that it will produce almost as much as the rice amount. The collection or the harvesting of the paddy is usually made by the early of August to September and the second season is around December to February. Table 1.1 proves the abundant availability of rice straw in Malaysia.

Table 1.1 : The Number of Land Being Planted With Rice and the Rice Straw Being Produced Preseason.

Project (State)	**Area (Hectare)**	**Amount Of Rice Straw (MT)**
Kedah and Perlis	60,359	301,759
Kelantan	35,973	179,865
Perak	36,354	181,770
Penang	10,138	50,690
Selangor	8,500	42,500
Total	151,324	756,584

*The number of the rice straw amount were calculated by counting the are the rice plantation area. (Sources: MADA, KADA and BERNAMA website)

1.5 Rationale and Significance

The rationale of using PHB instead of plastic is that it has the same physical properties as plyethylene but only brittle yet biodegradable (M. Flieger, 2002). *Bacillus cereus* is used instead of fungi because fungi is easily to be spread through out the environment. This bacteria is also widespread in nature and isolated from soil and growing plants (Kramer, 1989) thus, it is easily obtained in rotting food or any food waste. This is also due to its hydrolytic activities on food components (T.S.M. Pirttija¨rvi, 2000). It is also easy to sporulate on most media easily after 2 to 3 days (Granum, 2007). This bacteria is also capable to utilize cellulose in aerobic and moderate temperature condition (Rajvaidya *et* al., 2006). Rice straw is used due its availability and abundant stock in Malaysia as well as its cellulose content (MADA).

CHAPTER 2

LITERATURE REVIEW

2.1 Polymer

2.1.1 Plastics

Most plastics are made of polyethylene chain of monomer, methylene (CH_2). The chain is commonly as long as from 1000 to 10000 of repeated CH_2. Due its long chain, hence it is spoken of as high polymers or macromolecules (J. Brydson, 1999). Its molecular structure contributes to unbiodegradability of it. In soil, plastic will takes up to 1000 years to degrade. Whereas in water, it will take 450 years to degrade. During 1930s, plastics had reached comercial status: poly(vinyl chloride), polystyrene, poly(methyl methacrylate), low density polyethylene and nylon (Pasquini, 2005).

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 most used plastic nowadays is polyethylene and polypropylene (Pasquini, 2005). Polypropylene is a type of polymer that was a branched of low molecular weight oil and it was discovered in 1950.

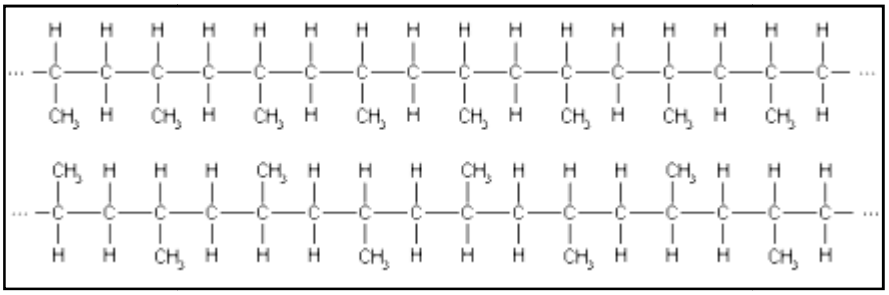

Figure 2.1 Molecular structure of polypropylene (Source: Absolute Astronomy, 2010)

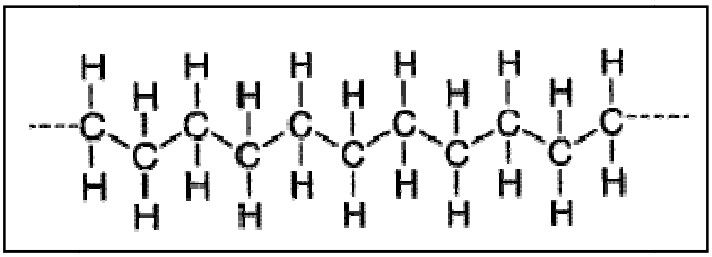

Figure 2.2 Molecular structure of polyethylene (Source: American Chemistry Council, 2010)

There are two types of plastic; thermoplastics and thermosets. Many of the polymers are thermoplastic. Thermoplastic is defined as it can be heated and reformed over and over again. This is important for easy processing and recycling. Unlike thermoplastic, thermosets cannot be reprocessed. If it is reheated, the material will scorch. Plastics can also be molded into bottles or anythong else as well as mixed with solvents to become an adhesive or paint. Plastics can deterioriate but can never decompose completely (American Chemistry Council, 2010)

Plastics are also applies to a wide range of materials that at some stage in manufacture are capable of flow such that they can be extruded, moulded, cast, spun or applied as a coating. Synthetic polymers are typically prepared by polymerization of monomers derived from oil or gas, and plastics are usually made from these by addition of various chemical additives. There are currently some 20 different groups of plastics, each with numerous grades and varieties. Plastics are incredibly versatile materials; they are inexpensive, lightweight, strong, durable, corrosion-resistant, with high thermal and electrical insulation properties. The diversity of polymers and the

versatility of their properties facilitate the production of a vast array of plastic products that bring technological advances, energy savings and numerous other societal benefits (Andrady & Neal 2009). The first truly synthetic polymer, *Bakelite*, was developed by Belgian chemist Leo Baekeland in 1907, and many other plastics were subsequently developed over the next few decades. It was not until the 1940s and 1950s, however, that mass production of everyday plastic items really commenced (Thompson, 2009)

2.1.2 Biopolymer

Plastic mentioned is a type of polymer, but synthetic polymer. It is because it is chemically derived from petroleum. Biopolymer are made biologically or naturally occured in the environment. Biopolymers are polymers that are generated from renewable natural sources, are often biodegradable and nontoxic. They can be produced by biological systems (such as microorganisms, plants and animals), or chemically synthesized from biological materials (such as sugars, starch, natural fats or oils) (Flieger *et al.*, 2002). To produce biopolymer chemically, it can be classified into three groups. Those are polyesters, polymers containing esther and other heteroatom-containing linkages in the main chain and also polyamino acids (Okada, 2002).

The common biopolymer in the environment is polysaccharide or known as starch. Starch is a major plant storage form of glucose. It consists of two components. Those are amylose, in which the glucose units are 1,4-α-D-linked together in straight chains, and amylopectin (can be identified by colored by elemental iodine) in which the glucose chains are highly branched. (Flieger *et al.,* 2002).

Table 2.1 : Some polysaccharides and their constituent monomers (Rastogi, 2003)

Polysaccharide	Monosaccharide units	Location and properties
Starch	D-glucose	Storage in plants
Mannans	D-Manose	Linear storage some higher plants
Cellulose	D-glucose	Structural polysaccharide in cell walls
Pectin	D-galactouronic acid	Fruits
Inulin	D-fructose	Linear polyfructosan in some plants
Glycogen	D-glucose	Branched storage polymer in animals

2.1.3 Polyhydroxybutyrate (PHB)

PHB is a type of biodegradable polymer (BDP), a family of polyhydroxyalkanoates (PHA) (Chen, 2005) and secondary metabolite (Rehm, 1997). Biodegradation is a process that breaks down a xenobiotic into simpler form. Ultimately, the biodegradation of organics results in the release of carbon dioxide (CO_2) and water (H_2O) to the environment (G. Landis *et al.*, 2003). The most commonly studied PHA, polyhydoxybutyrate (PHB), is known to possess physical properties similar to those of polyethylene, and has potential applications as disposable bulk material in packing films, containers, or paper coatings, amongst others (Quillaguamán *et al*., 2007).

PHB is an energy reserve polyester naturally accumulated by a wide variety of microbes. PHB-like copolymer is PHBV (polyhydroxybutyrate valerate). It is less stiff and tougher thus used as packaging material. Melting point of PHB is 40 -180 ºC. PHB has the same properties as polypropylene but stiffer and brittle. PHB also degradable in microbially active environments from 5 to 6 weeks (Shimao, 2001). The mode of action to be degraded is the enzyme from bacteria degrade the PHB.

Then it is absorbed through the cell wall and metabolized. If it is aerobic condition, it will be degraded to CO2 and H2O. But, in anaerobic condition, it is degraded to methane, CO2 and H2O (Lieger, 2002).

2.1.3.1 PHB Molecular Structure

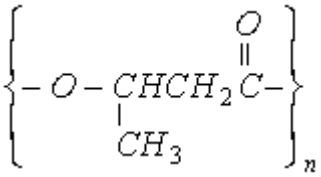

Figure 2.3 Molecular structure of PHB (Maia *et al.,* 2004)

PHB is the most common member of PHA. It belongs to to the short chain length PHA (scl PHA) with its monomers constitute of 4 to 5 carbon atoms. It is usually brittle but has a thermoplastic characteristic (Chen, 2005).

2.1.3.2 PHB as Secondary Metabolite

Secondary metabolite is the a byproduct synthesize by microorganism, not the major product and it increased greatly when substrates depleted from the medium (Rehm *et al.*, 1997). PHB is secondary metabolite because it is synthesize by microbes when there is no sufficent nutrient to promote their growth, but excess in carbon source. IT does not play essential role in development of the producing organisms, but convey advantages to the pertinent species concerning its long term survival in the biological community anda environment (Rehm *et al.*, 2997). Maximum production is when growth promoting substrates were depleted from the medium and this phenomenon is calle ‘catabolic regulation’ (Rehm *et al.*, 1997).

The causes of PHB may be produced as secondary metabolite are:

a) Nutritional downshift in the media caused by limitation of particular metabolites promote excessive formation of some metabolite due to an imbalanced metabolism. These accumulates or precursor is known to induce secondary pathways.
b) Limitation of some endegenous metabolites could be important which inhibit global regulatory mechanisms governing aerial mycelium and spore formation. Thus in this repressing or inhibitory effects on the secondary pathways and on morphogenesis could be diminished. (Rehm *et al.,* 1997)

2.1.3.3 Process Synthesis of PHB

PHB is synthesized by acetyl-CoA by the sequencial action of 3 enzymes; B-ketothiolase (phbA), acetyl-CoA reductase (phbB) and PHB polymerase (phbC). Production of PHB in plant is based on consumption of acetyl-CoA (initial substance). So, oil crops is a potential target for production of PHB due to its high flux of acetyl-CoA during the oil synthesis stage (Redd *et al.,* 2003; Omidvar *et al.,* 2008).

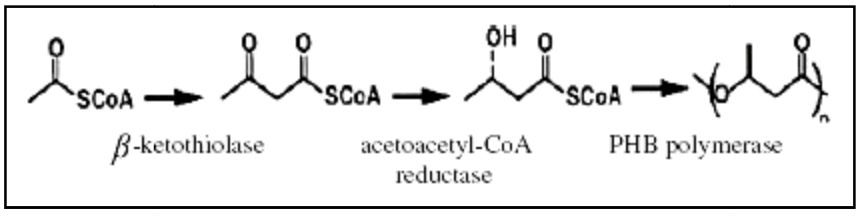

Figure 2.4 Polyhydroxybutyrate (PHB) biosynthetic pathway. PHB is synthesized by the sequential action of b-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB), and PHB polymerase (phbC) in a three-step pathway (Madison and Huisman 1992)

To produce PHB, theoretical yields of PHB from several carbon sources have been estimated from biochemical pathways leading to PHB. In estimating the yields,

a special emphasis is made on recycling (or regeneration) of $NADP^+$ which is the co-substrate of acetoacetyl-CoA reductase, one of three key enzymes involved in the biosynthesis of PHB. As a $NADP^+$-regenerating enzyme, glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase is conceived (Yamane, 1992). Figure 2.4 illustrates how PHB can be synthesized by metabolism of bacteria: The pathway to produce PHB is as in Figure 2.5.

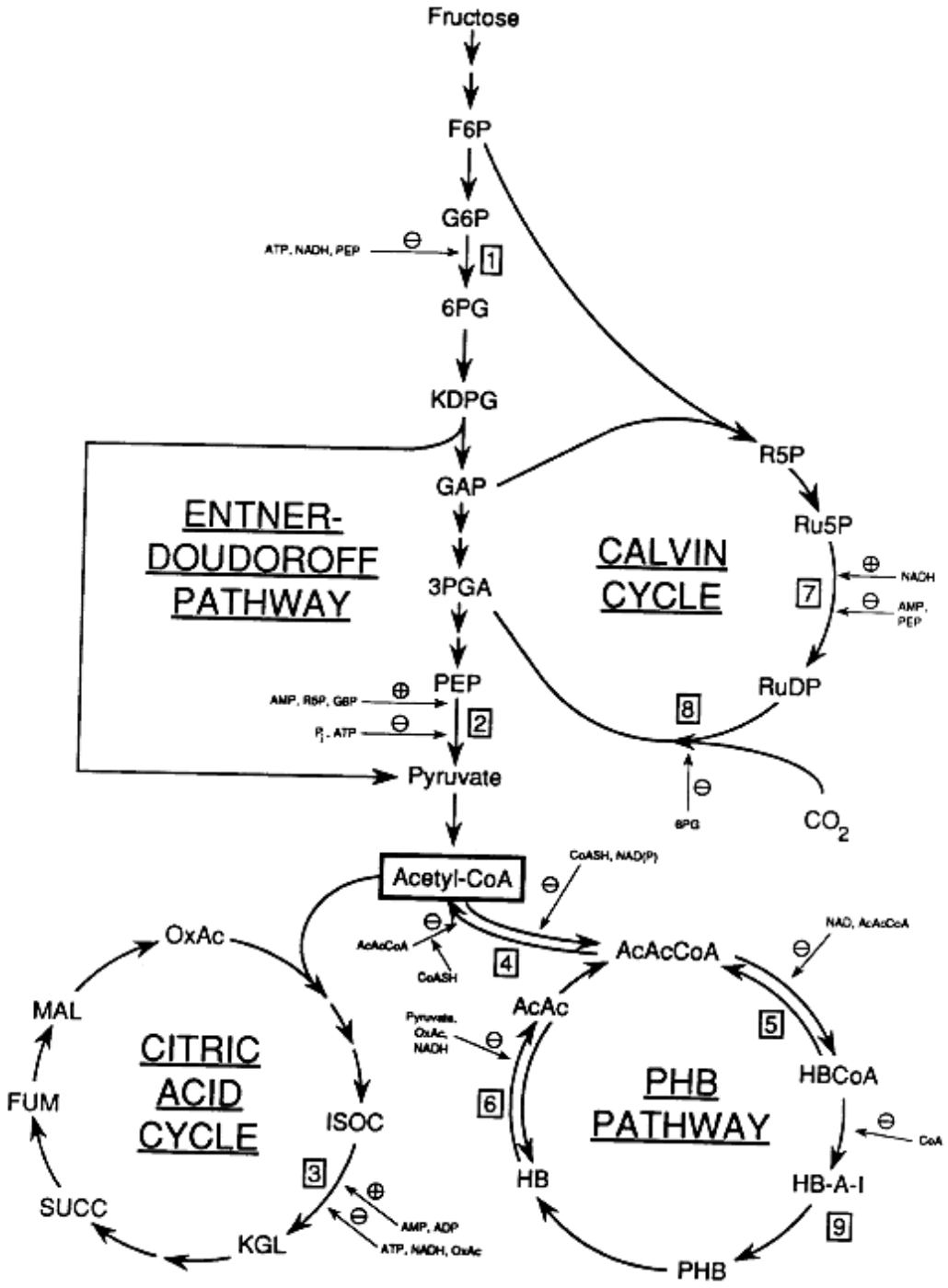

Figure 2.5 Metabolic pathway to produce PHB (Guske, 1990)

2.2 Carbon Source

Carbon source that is used in this paper is cellulose from rice straw. *B.cereus* will utilise cellulose for energy and growth. This is due to the fact that the bacteria needs carbon source to continue its system. Rice straw is used as second generation energy source instead of using first generation due to humanity issues (such as starvation).

So, before proceeding to the experiments, cellulose content and its availability in rice straw is investegated. Comparison between the content in the rice straw and other wastes is also observed.

2.2.1 Rice Straw

The chemical composition in rice straw plays the major role as a substrate for PHB production. In rice straw, there is not only cellulose available, but also contains other carbon based elements. One of the major carbon elements is hemicellulose.

Table 2.2 summarizes the contents of cellulose and hemicellulose available in the rice rice straw:

Table 2.2 : Carbon source content in rice straw (Zhu *et al.,* 2005)

Carbon source	Content (%)
Cellulose	38.6
Hemicellulose	19.7

Whereas Table 2.3 is the list of chemical properties contain in three types of plant wastes: rice straw, rice husk and wheat straw to highlight the particulate differences in feedstock as below:

Table 2.3 : Proximate composition and selected major elements of ash in rice straw, rice husk and wheat straw (Jenkins *et al.,* 1998)

	Rice straw	**Rice husk**	**Wheat straw**
Proximate analysis (% dry fuel)			
Fixed carbon	15.86	16.22	17.71
Volatile matter	65.47	63.52	75.27
Ash	18.67	20.26	7.02
Total	100.00	100.00	100.00
Element composition oof ash (%)			
SiO_2	74.67	91.42	55.32
CaO	3.01	3.21	6.14
MgO	1.75	<0.01	1.06
Na_2O	0.96	0.21	1.71
K_2O	12.30	3.71	25.60

Rice straw feedstock has low total alkali content (Na_2O and K_2O comprise < 15% of total ash) whereas wheat straws have < 25 alkali content in ash (Baxter et al., 1996). Rice husk which is also of poor feed quality, caused mainly by high silica. The fixed carbon shows that rice straw has 15.86 % of cellulose and other major carbon substance.

2.2.2 Carbon Source: Cellulose

Plant biomass consists of three main polymeric components. Those are cellulose, hemicellulose and lignin. In softwoods, hardwoods and the abundantly available agricultural residues such as wheat, rice and other cereal straws, sugarcane bagasse, corn stalks, corncobs, jute and cotton stalks, cellulose is the chief constituent (over 40 % by weight), then hemicellulose (~ 30 %) and lignin (~ 20%).

This shows that cellulose is the most abundant compound on earth, followed by hemicellulose and lignin (Varma, 2005).

Cellulose is very resitant to hydrolysis. This is due to the straight chain of B-1,4-linked glucose units without any side chains. Thus, extensive hydrogen bonding between the cellulose molecules that forms crystalline structure. At least, three different enzymes are required for the complete hydrolysis of the crystalline. Those are endo-ß-glucanase, exo-ß-glucanase and cellobiase (Kim, 2008).

2.2.2.1 Molecular Structure of Cellulose

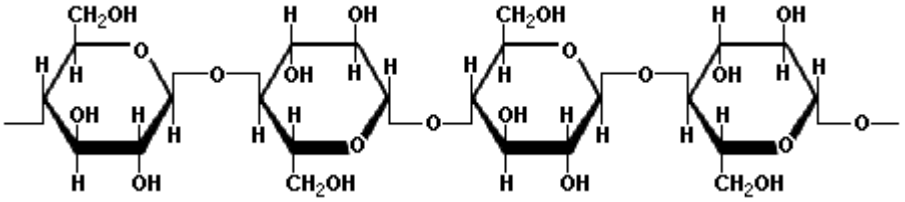

Figure 2.6 Molecular structure of cellulose (Sientific Psychic, 2005)

It is the major structural compound of cell wall of higher plants. Cellulose is also a high molecular weight linear polysaccharides of D-glucopyranose monomer units that joined together by B-1→ 4 linkage or also known as B-(1→4) glucosidic linkage. The B(1→4) adopt a fully extended conformation in which the glucose units zig zag along the polymeric chain (Tsai, 2007). Pickering (2000) also stated that cellulose cell wall provides structural support. This is due to the pressure of cell contents leads to turgidity thus may break the cell wall if no cellulose present. Osmotic intake of water can can damage the cell. Thus, cellulose is important to protect it and made the cell wall permeable to water and other dissolved substances.

According to Rastogi (2003), monomer of cellulose is D-glucose and in the plant cell wall, it is associated with lignin. Cellulose may be hydrolysed to its monomers. The process is called hydrolysis. There are two types of hydrolysis

process. Those are chemical and biological hydrolysis. For biological hydrolysis, the hydrolyse element is the reaction of the enzyme from the bacteria (such as cellulase). Whereas chemical hydrolysis uses two ways two do the actions. They are dilute acid hydrolysis or concentrated acid hyrolysis. Strong acid that is commonly use due to its high conversion is sulfphuric acid. Diluted acid hydrolysis is using diluted as hydrolyser (Rastogi, 2003). These reactions has different products eventhough the objective is the same. The summary of the reaction is:

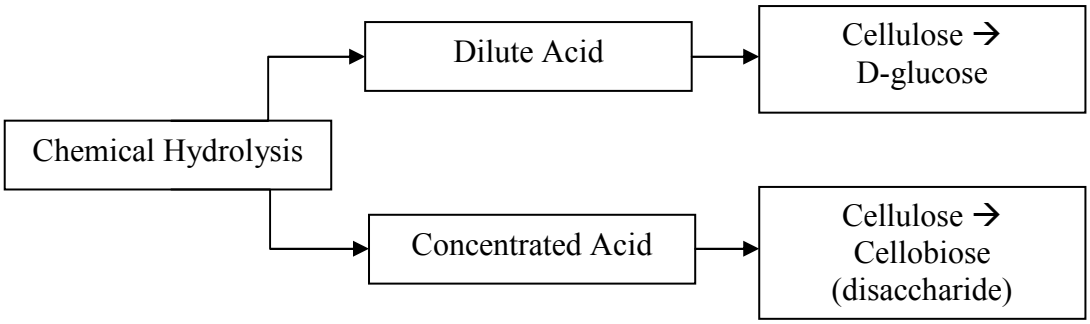

Figure 2.7 Summary reaction of chemical hydrolysis (Rastogi, 2003)

2.2.2.2 Degradation of Cellulose by Bacteria

Three enzymes react on degradation of cellulose are C_1, Glucanase or Cx and β-glucosidase. (Rajvaidya *et al*., 2006)

C1 acts on native cellulose, then glucanase cleave the partially degraded cellulose. Thus, cellobiose and oligamers are formed. Glucanase claeve the bonds between glucose units at random. Lastly, β-glucosidase hydrolyze the cellobiose and oligomers into glucose. The regulation of this process is regulated by a regulatory mechanism called catabolic repression (Rajvaidya *et al*., 2006).

2.2.3 Availability of Rice Straw

Potential of availability of rice straw is very high because the plantation of the rice will yield 0.45: 0.55 for rice straw with rice itself. This ratio were calculated by assuming that every hectare of paddy field will produce 4 to 5 tone of rice and that mean that it will produce almost as much as the rice amount. The collection or the harvesting of the paddy is usually made by the early of August to September and the second season is around December to February. Usually the second season weather were more dried and sunny which will produce and yield a lot of rice straw. Believe, the second season can produce also double of the amount compared to the first season.

The government also has conducted a few paddy plantation projects such as in Kedah under MADA project, Kelantan under KADA project and also Kerian, Penang and Seberang Perai under IADP project (Integrated Agricultural development Project). These projects are believed help the number of the rice straw available in Malaysia quite high. In Selangor also have a small amount of rice plantation but the number is not very significant.

Table 2.4 : The Number of Land Being Planted With Rice and the Rice Straw Being Produced Preseason.

Project (State)	**Area (Hectare)**	**Amount Of Rice Straw (MT)**
Kedah and Perlis	60,359	301,759
Kelantan	35,973	179,865
Perak	36,354	181,770
Penang	10,138	50,690
Selangor	8,500	42,500
Total	151,324	756,584

*The number of the rice straw amount were calculated by counting the are the rice plantation area. (Sources: MADA, KADA and BERNAMA website)

2.3 Delignification

Delignification is a process where lignin is degraded by enzymatic reaction of certain bacteria or other chemical process. Occurence of lignin slows the rate of cellulose decomposition due to physical effect resulting from close structural inter linkage between cellulose and lignin in the cell wall (Rajvaidya *et al.*, 2006). In this paper, reaction of *B.cereus* is considered. Delignification is crutial because its chemical durability makes it indigestible to organisms because of its bonding to cellulose and protein material. Lignin fills up the spaces between the polysaccharides fibers acting to cement them together. This lignin sheet acts as a barrier towards the outside elements (Carraher, 2008). Thus, if it is not degraded first, the metabolism of *B.cereus* towards cellulose will be hindered because of the lignin 'gates' or 'walls'.

2.3.1 Lignin

Lignin is a highly branched polymer that makes cell walls more rigid. It is third most abundant structural polymeric material found in plant cell walls (comprising about 20 to 30 % of woody biomass) (Turley, 2008) that have supporting or mechanical function. Some plants have lignin deposited in primary and secondary cell walls (Raven *et al.,* 1996). Lignin binds hemicellulose and cellulose together in plant cell walls thus shields them from enzymatic and and chemical degradation (Turley, 2008).

In addition, Carraher (2008), mentioned about lignin in his book '*Polymer Chemistry'*. After the saccharides, lignin is the second most widely used produced organic material. It is found that it is essentially in all living plants and the major noncellulosic constituent of wood. Lignin has a complex structure that varies with the source, growing conditions, etc. This complex abd varied structure is typical of many plant-derived macromolecules. Lignin is generally considered as being formed from three different phenylpropanoid alcohols (coniferyl, coumaryl and sinapyl

alcohols) which are synthesized from phenylalanine via various cinnamic acid derivatives.

It is usually found in plant cell walls of supporting and conductiing tissue, mostly the tracheids and vessel parts of the xylem. It is largely found in the thickened secondary wall, but can occur elsewhere close to the cellulose and hemicellulose.

2.3.2 Molecular Structure of Lignin

Lignin is the main non-carbohydrate polymer found in plants. It is very complex, highly aromatic, polyphenolic material with a complex, cross-linked structure derived from coniferyl alcohol (Turley, 2008). Due to lignin's contents of rigid aromatic groups and hydrogen bonding by the alcohol, aldehyde and ether grioups gives a fairly rigid material that strengthens stems and vascular tissue in plants, allowing upward growth (Carraher, 2008). It is compatible to cellulose because it contains carbonyls and unsaturation, phenols and carbohydrates (Gnanou, 2008).

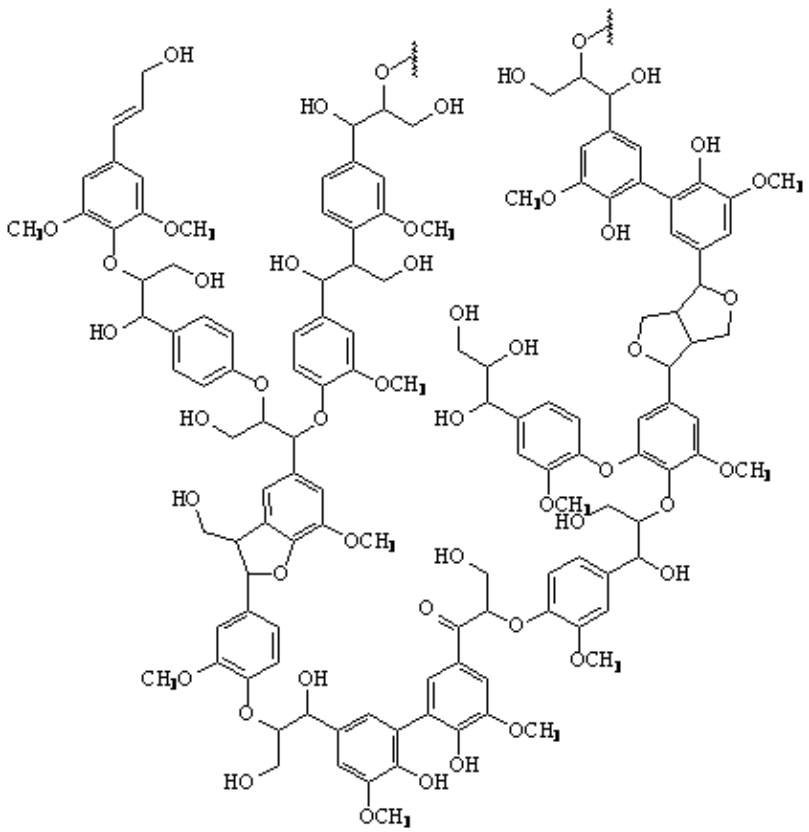

Figure 2.8 One of molecular structure of lignin (Gregory, 2007)

Lignin's heterogenous structure consists interphenylpropane-unit linkages of carbon-carbon and carbon to oxygen, side chains with various substituent and functional groups (Agarwal, 2008). The linkages between lignin and cellulose is believed to be phenyl glycoside bonds, benzyl esters and benzyl esthers (Balakshin *et al.,* 2008).

Lignin also allows water and minerals to be conducted through the xylem under negative pressure without collapsing the plant. This structure can be flexibilized through introduction of plactisizer. In nature, the plactisizer is namely, water. The presence of hydrophilic aromatic groups helps ward off excessive amount of waters allowing the material to have a variable flexibility but to maintain some strength. Without the presence of water, lignin is brittle but with water the tough lignin provides plants with some degree of protection from animals.

2.3.3 Degradation of Lignin Process

To degrade, the lignin must be solubilized first. It is insoluble but can be solubilized by strong basic solutions that can disrupt the internal hydrogen bonding. After solubilized, it is easier to be oxidized and the presence of aromatic units containing electron withdrawing ether and alcohol moieties makes it available for electrophilic substitution reactions (Carraher *et al.,* 2008). Some oxidizers are such chlorine or chlorine dioxide (Bezalel *et al.*, 1993). But, most of the chlorine based chemicals oxidize the aromatic lignin compounds, some of the chlorine enters the ring by substitution reactions. The aromatic chlorine compound is not readily biodegraded, thus present problem towards the environment (Bezalel *et al.,* 1993). This triggers the investigation towards biologically degrade the lignin. Table 2.9 summarizes the delignification process.

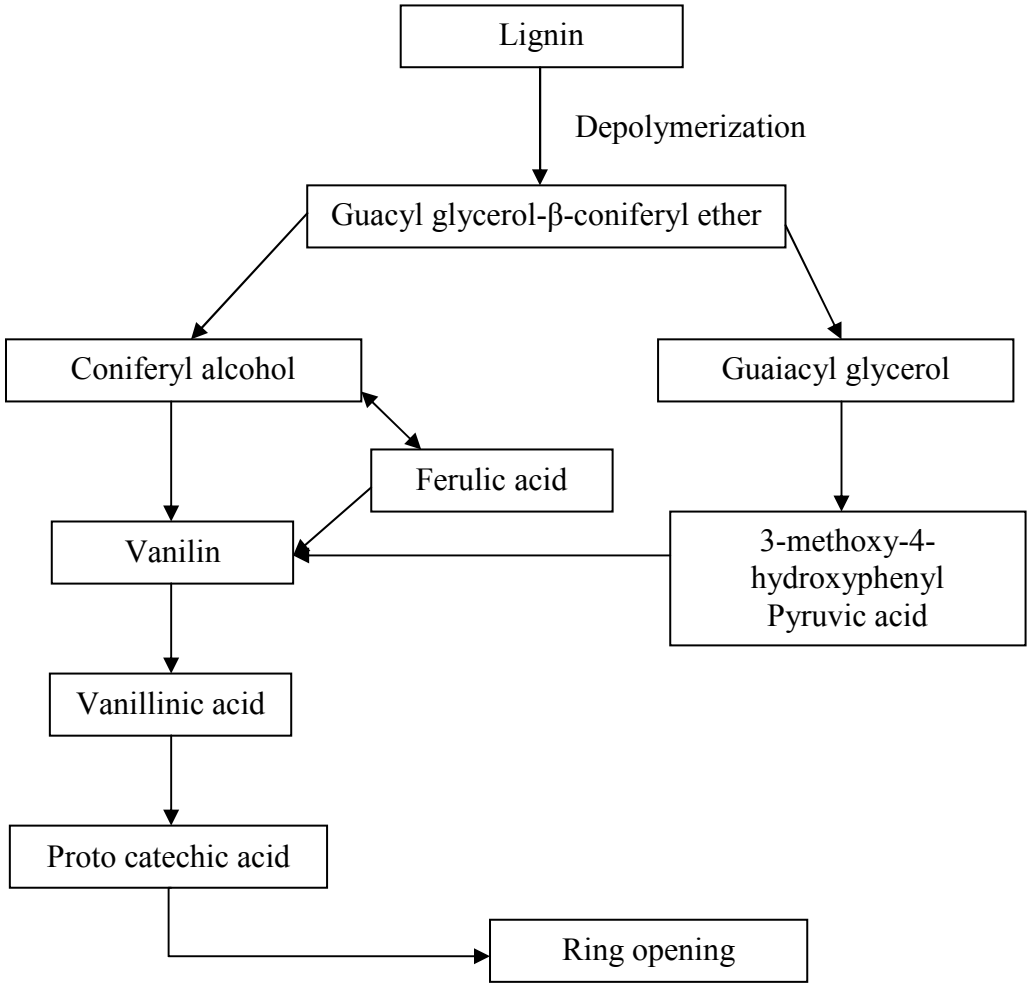

Figure 2.9 Lignin decomposition (Rajvaidya *et al*., 2006)

2.3.3.1 Types of Delignification Process

Delignification is carried out in acidic or basic conditions and in the presence of sulfur in various forms. It results primarily from a rupture of –C-O-C- bonds. The types of process: (Gnanou *et al.,* 2008)

a) Kraft process:

   The wood shavings treated at 170 ºC under pressure during a few hours in the reactor that has aqeous solution of soda and sodium sulfide. The resulting hydrolysis allows extraction of a black liquid where the lignin components are recovered by precipitation through modulation of the concentration and pH. (Gnanou *et al.,* 2008)

b) Lignosulfonate process:

   The wood is treated by a sulfide (sodium, calcium, ammonium or magnesium sulfite) which generates SO_2 *in situ*. Then, reacts with lignin simultaneously and brings about acid hydrolysis that induces the degradation of the network and generates highly water soluble lignosulfonates that can be separated from cellulose. (Gnanou *et al.,* 2008)

c) Other mechanisms:

   For example, flash self-hydrolysis that results from the explosion of shavings of wood impregnated with steam under pressure. (Gnanou *et al.,* 2008)

d) Enzymatic:

   The enzymes that degrade lignin are called lignin modifying enymes. Those are peroxidases, dehydrogenases, hydrogen peroxide and oxidases. (Viikari *et al.,* 2001).

   In other words, to degrade lignin, it must undergo oxiadative reactions for lignin dipolymerization. White-rot fungi are the most efficiently degrade lignin from wood (Madras, 2005). Nonfilamentous bacteria mineralize less than 10% of lignin preparations and can degrade only teh low molecular weight part of lignin as well as degradation products of lignin (Hatakka, 2001).

2.3.3.2 Enzymatic Deglinification

2.3.3.2.1 Lignin Peroxidase

The reaction sequence:

$$LiP\ (Fe^{3+}) + H_2O_2 \rightarrow LiP\text{-}I\ (Fe^{4+}\text{-}O)\ P + H_2O$$
$$LiP\text{-}I\ (Fe^{4+}\text{-}O)\ P + R \rightarrow LiP\text{-}II\ (Fe^{4+}\text{-}O)\ P + R$$
$$LiP\text{-}II\ (Fe^{4+}\text{-}O)\ P + R + 2H^{+} \rightarrow LiP\ (Fe^{3+})\ P + R + H_2O$$

R is an aromatic substrate or lignin substructure, P is Porphyrin, LiP carries oxidizing equivalents to H_2O_2, one asoxyferryl centre and one as a porphyrin π cation radical (P). In the presence of H_2O_2 LiP is readily converted to catalytically inactive intermediate. (Hatakka, 2001)

2.3.3.2.2 Manganese Peroxidase

The reaction sequence:

$$MnP\ (Fe^{3+})\ P + H_2O_2 \rightarrow MnP\text{-}I\ (Fe^{4+}\text{-}O)\ P + H_2O$$
$$MnP\text{-}I\ (Fe^{4+}\text{-}O)\ P + Mn^{II} \rightarrow MnP\text{-}II\ (Fe^{4+}\text{-}O)\ P + Mn^{III}$$
$$MnP\text{-}II\ (Fe^{4+}\text{-}O)\ P + Mn^{II} \rightarrow MnP\ (Fe^{3+})\ P + Mn^{III} + H_2O$$

P is porphyrin, MnP-I carries both oxidizing equivalents of H_2O_2 carries only one axidizing equivalent. Mn^{3+} oxidizes phenolic rings in lignin and and lignin model compounds to phenoxyl radicals that leads to decomposition of these structures. (Hatakka, 2001)

2.3.3.3 Previous Research on Biodegradation of Lignin

A study had been done by Bezalel *et al* (1993) regarding biologically degrading the lignin by using *Bacillus stearothermophilus*. The reaction of the microbe and lignin is described; An o~-L-arabinofuranosidase would be expected to split the oM,3-glycosidic bond between arabinose and xylose and release a lignin-O-L-arabinose (lignin connected by an ether bond to L-arabinose). The enzyme would have the potential to delignify pulp and to act synergistically with xylanases on the arabinoxylan hemicellulose. Furthermore, it could remove the lignin without removing much of the hemicellulose because arabinose is a side chain of the main xylan polymer. From the result obtained, The presence of mannose, glucose or xylose in the medium resulted in a higher extracellular delignification activity than when no carbohydrate was added.

Another research has been done by Xin Li *et al.* (2002). It has been demonstrated that the use of white-rot fungi helps remove lignin from lignocellulosic materials. When several white-rot fungi, including *Phlebia* sp. MVHC 5535, were employed to degrade long fiber bagasse (LFB) (Breccia *et al.*), they produced LFB with the lowest lignin content, but the highest delignification efficiency was less than 17% after 30 days of incubation. 6 *Phlebia* sp. MG-60 removed more than 21.9% of lignin in LFB, so it can be regarded as an effective strain for biopulping or biodegradation of lignocellulosic residues. Although pulp property of the decayed bagasse was not analyzed to evaluate its pulping possibility, it should be helpful during mechanical or chemical pulp production by selectively removing as much lignin as possible during the fungal pretreatment process (Xin Li *et al.,* 2002).

Ratto *et al.* (1993), has investigated the delignification by *Dictyoglomus* sp. Enzymatic treatment of pine and birch kraft pulps with a xylanase preparation from a thermophilic anaerobic bacterium *Dictyoglomus* sp. strain B1 was studied in order to improve pulp bleachability. Maximal solubilization of pulp xylan was obtained at 90°C and pH 6.0-7.0. The enzyme was also active in the alkaline pH range; at pH 9.0 xylan hydrolysis was decreased by only 18% from the maximum at pH 7.0. The

positive effect of xylanase pretreatment at 80°C and pH 6,0 or 8.0 on bleachability of pine kraft pulp was demonstrated. The brightness was increased by two ISO units in one-stage peroxide delignification, which corresponds well to values obtained with other enzymes at lower temperatures and pH values. Thus, the *Dictyoglomus* xylanase is well suited for pulp treatments at elevated temperatures in neutral and alkaline conditions (Ratto *et al.,* 1993).

2.4 *Bacillus cereus*

B.cereus is a spore forming bacteria, Gram positive, aerobic or facultatively anaerobic. It occurs in different foods such as milk, vegetables, spices, rice and sauces (Chorin *et al*., 1997). Its pathogenicity is linked to the production of two toxins; thermostable emetic enterotoxin (Jonhnson, 1984). And a thermosensitive diarrhoegenic enterotoxin (Goepfert *et al.,* 1972). It was investigated that more than 10^5 bacteria per gram is needed for intoxication to occur (Johnson, 1984).

Its ability to sporulate makes it quite resistant to heat treatments (Chorin *et al.,* 1997). *B.cereus* causes a toxin-mediated food poisoning. *B.cereus* is an aerobic and facultatively anaerobic, spore-forming, gram-positive bacillus. The emetic syndrome is caused by a preformed heatstable toxin. The diarrhea syndrome is caused by in vivo production of a heat-labile enterotoxins (Louisiana Office of Public Health, 2004).

2.4.1 Detection and Isolation of *B.cereus*

Official detection methods for this microbe are based on biochemical properties and primarily on positive lechitinase reaction (Pirttijarvi *et al.*, 2000). *B.cereus* is closely related to *B.antharacis, B.megaterium, B.thuringiensis.* But,

B.cereus can be distinguished from these species by biochemical tests and the absence of toxin crystals (Setlow *et al.*, 2007).

Some techniques are applicable such as by using a non-selective medium of blood agar (sometimes with the addition of polymyxin as a selective agent to surpress Gram-negatives). *B.cereus* can be identified after 24 hours of incubation at 37º C incubation by its characteristic colonial morphology of large (3 to 7 cm diameter), flat or slightly raised, grey-green colonies with a characteristic granular or ground-glass texture and a surrounding zone of α or β haemolysis. (Adams *et al.,* 2008). This can be observed in Figure 2.10.

To confirm the identity of a blood agar isolate or to isolate smaller numbers of *B.cereus* from foods, a more selective diagnostic agar is necessary. Polymyxin/pyruvate/eggyolk/mannitol/bromothymol blue agar (PEMBA) is one of the examples. On PEMBA, *B.cereus* produces typical crenated colonies which retain the turquoise-blue of the pH indicator (bromothymol blue) due to their inability to ferment mannitol, they are surrounded by a zone of egg-yolk precipitation that is caused by lecithinase activity. (Adams *et al.,* 2008)

Colonies of *B.cereus* can be confirmed by a microscopic procedure combining a spore stain with an intracellular lipid stain. Spores appear green in a cell with red vegetative cytoplasm and containing black lipid globules. Biochemical confirmation can be based on an isolate's ability to produce acid from glucose but not from mannitol, xylose or arabinose (Adams *et al.,* 2008). This can be observed in Figure 2.11.

Figure 2.10 *B.cereus* colonies on blood agar (Todar, 2008)

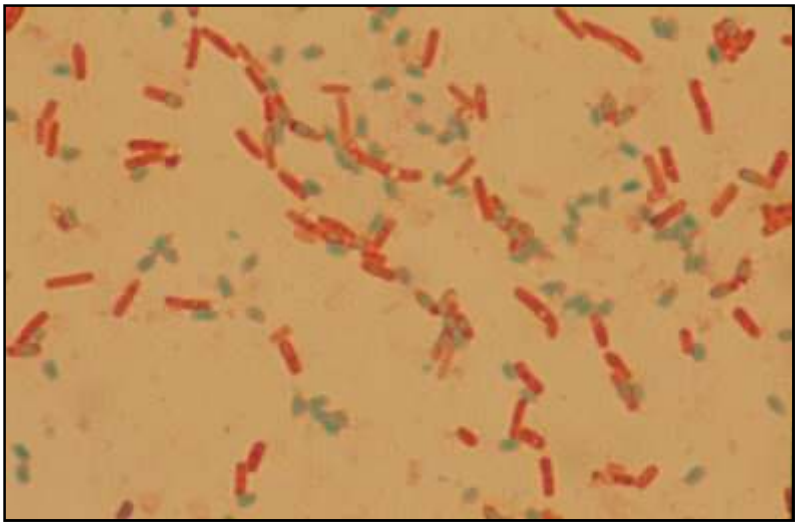

Figure 2.11 *B.cereus* spore stain (Todar, 2008)

2.4.2 Characteristics

It is Gram-positive, aerobic, spore forming rods, sometimes display a Gram-negative or variable reaction. Over the past 30 years, In *B.cereus* group, there are 6 species. Those are *Bacillus anthracis, B.cereus, Bacillus mycoides, Bacillus pseudomycoides,* and *Bacillus weihenstephanensis* (Granum, 2007). *Bacillus* has expanded to acommodate more than 100 species (Ash, 1992). *B.cereus* has 16S and

23S rRNA sequence. *Bacillus* species are easily sporulate on most media from 2 to 3 days (Granum, 2007). *B.cereus* is closely related to *B. Antharacis, B.megaterium, B.thuringiensis*. But, *B.cereus* can be distinguished from these species by biochemical tests and the absence of toxin crystals (Setlow *et al.*, 2007).

B.cereus is facultatively anaerobic with large vegetative cells. The siza varies from 1.0 μm by 3.0 to 5.0 μm in chains. It does not have any marked tolerance for low pH (min 5.0 – 6.0, depends on the acideulant) or water activity (~min 0.95) (Adams *et al.,* 2008).

The spores are central, ellipsoidal in shape and do not cause in swelling in the sporangium. As a spore former, it is widely distributed in the environment and can be isolated from soilm water and vegetation. This equibity means that it is also a common component of the transient gut flora in humans. The spores are heat resistance (Adams *et al.,* 2008).

2.4.3 Growth Condition

Its spores can spread widely in foods and are commonly found in milk, cereals, starches, herbs, spices and other dried foodstuffs. It can also be found on the surfaces of meat and poultry. This is due to dust and soil contamination as well as the spores' hydrophobic and ability to attach to food surfaces (Husmark, 1992). The spores are ellipsoidal and central to subterminal and do not distend the sporangium. Spore germination can occur over the temperature range of 8º to 30º C.

Tha bacteria can grows over the temperature range of approximately from 10º to 48 ºC. The optimum temperature is between 28º to 35ºC (Setlow *et al.,* 2007; Adams *et al.,* 2008). For water activity, the minimum required is 0.95. Whereas pH, it grows in a range of 4.9 to 9.3. But the conditions for water activity and pH are

dependent with each other including temperature and the interrelated parameters (Setlow *et al.,* 2007).

Table 2.5 : Growth requirements of sporeformers of public health significance (Setlow *et al.,* 2007)

Organism	Inhibitory condition		Temperature range for growth (ºC)
	Minimum pH	NaCl conc (%)	
Group I *C.botulinum*	4.6	10	10 – 50
Group II *C.botulinum*	5.0	5	3.3 – 45
B.cereus	4.35 – 4.9	ca. 10	5 – 50
C.perfringes	5.0	ca. 7	15 – 5

Table 2.3 shows the minimum requirements for inhibitory of growth of the 4 bacteria, including *B.cereus*. The pH to inhibit the sporeformation is from 4.35 to 4.9. So, in this paper, neutral pH is used to ensure the growth is not inhibited. The temperature for growth is between 5º to 50º C. So, in the research, optimum temperature of 30º C is used.

2.4.4 Reservoirs

It is widespread in nature and frequently isolated from soil and growing plants (Kramer, 1989). From this natural environment, it is easily spread to foods especially from plant origin. Research showed that *B.cereus* frequently present in raw materials and ingredients used in the food industry such as vegetables, starch and spices (Granum, 2007). It can contaminate milk and milk products if it is in contact with soil and grass from udders of cows. The spores can survive milk pasteurization and after germinate, the cells are from competition from most other vegetative cells (Andersson, 1998). According to Granum (2007), Claus (1986) stated that *B.cereus* is unable to grow in milk and milk products that is strored 4 to 8 ºC.

2.4.5 Illnesses

B.cereus has been recognized as an agent of food poisoning since 1955. There are only a few outbreaks a year reported by CDC. Between 1972 and 1986, 52 outbreaks of food-borne disease associated with *B. cereus* were reported to the CDC (in 2003, there were two), but this is thought to represent only 2% of the total cases which have occurred during these periods. It is not a reportable disease, and usually goes undiagnosed. The bacteria must grow to very high numbers (> 10^6 /g of food) to cause human illness (Setlow *et al.,* 2007).

B. cereus causes two types of food-borne illnesses. One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. It resembles *Staphylococcus aureus* (staph) food poisoning in its symptoms and incubation period. This is the "short-incubation" or emetic form of the disease.

The second type is manifested primarily by abdominal cramps and diarrhea following an incubation period of 8 to 16 hours. Diarrhea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or diarrheal form of the disease, and it resembles food poisoning caused by *Clostridium perfringens.* In either type, the illness usually lasts less than 24 hours after onset. In a few patients symptoms may last longer.

The short-incubation form is caused by a preformed, heat-stable emetic toxin, ETE. The mechanism and site of action of this toxin are unknown, although the small molecule forms ion channels and holes in membranes. The long-incubation form of illness is mediated by the heat-labile diarrheagenic enterotoxin Nhe and/or hemolytic enterotoxin HBL, which cause intestinal fluid secretion, probably by several mechanisms, including pore formation and activation of adenylate cyclase enzymes. (Todar, 2008)

2.4.6 Toxins

B.cereus produces one emetic toxin (ETE) and three different enterotoxins: HBL, Nhe,and EntK.

Two of the three enterotoxins are involved in food poisoning. They both consist of three different protein subunits that act together. One of these enterotoxins (HBL) is also a hemolysin; the second enterotoxin (Nhe) is not a hemolysin. The third enterotoxin (EntK) is a single component protein that has not been shown to be involved in food poisoning. All three enterotoxins are cytotoxic and cell membrane active toxins that will make holes or channels in membranes.

The emetic toxin (ETE) is a ring-shaped structure of three repeats of four amino acids with a molecular weight of 1.2 kDa. It is a K^+ ionophoric channel, highly resistant to pH between 2 and 11, to heat, and to proteolytic cleavage.

The nonhemolytic enterotoxin (Nhe) is one of the three-component enterotoxins responsible for diarrhea in *B.cereus* food poisoning. Nhe is composed of NheA, NheB and NheC. The three genes encoding the Nhe components constitute an operon. The *nhe* genes have been cloned separately, and expressed in either *Bacillus subtilis* or *Escherichia coli*. Separate expression showed that all three components are required for biological activity.

The hemolytic enterotoxin, HBL, is encoded by the *hbl*CDA operon. The three protein components, L1, L2 and B, constitute a hemolysin. B is for binding; L1 and L2 are lytic components. This toxin also has dermonecrotic and vascular permeability activities, and it causes fluid accumulation in rabbit ileal loops. (Todar, 2008)

CHAPTER 3

METHODOLOGY

This chapter will be discussing regarding the 4 main processes of this research; growth curve of *B.cereus,* delignification of the rice straw, production and analysis of PHB.

3.1 Feedstock Material

Rice straw from Nibong Tebal paddy field was used in this research. The rice straw was milled in a hammer mill to ensure the increase in surface area for the reaction of *B.cereus* with the rice straw. Then, the rice straw was air dried in a tray drier for 3 days at 40 ºC.

3.2 Preparation of Seed Culture

3.2.1 Microorganism

The bacteria to be used was *B.cereus*.

3.2.2 Subculturing *B.cereus*

3.2.2.1 Agar Preparation

Agar was prepared by using basis of 1 L water. The chemical used for the agar were 10 g of peptone, 2 g of yeast extract, 1 g of $MgSO_4.7H_2O$ and 15 g of agar powder. The pH was maintained at pH 7. The 1 L of agar solution was prepared in 2 L Schott bottle.

First, the bottle was filled with 1 L of distilled or diionized water and put on a hot plate until boiled. Magnetic stirrer was added to ensure the homogeneity of the agar. After the water was boiled, 10 g of peptone, 2 g of yeast extract, 1 g of $MgSO_4.7H_2O$ and 15 g of agar powder were added one by one. The agar was ready when the medium was homogenized. The homogeneity was determined by observing the condition of the liquid agar; absent from solid particles.

After that, the agar solution was autoclaved for 20 minutes at 121 ºC. Then, the liquid agar was brought under laminar flow to prevent contamination. The agar solution was poured in petri plates and left until solidifies while still under the flow.

After that, the agar are sealed with parafilm inside a plastic bag and stored under 4 ºC medium refrigerator in inverted manner to ensure no vapourization occured in the petri dish.

3.2.2.2 Broth Preparation

Broth was prepared by using basis of 1 L water. The chemical used were 10 g of peptone, 2 g of yeast extract and 1 g of $MgSO_4.7H_2O$. The pH was maintained at pH 7.2. The 1 L of agar solution was prepared in 2 L Schott bottle.

First, the bottle was filled with 1 L of distilled or diionized water and put on a hot plate until boiled. Magnetic stirrer was added to ensure the homogeneity of the agar. After the water was boiled, 10 g of peptone, 2 g of yeast extract and 1 g of $MgSO_4.7H_2O$ were added one by one. The agar was ready when the medium was homogenized. The homogeneity was determined by observing the condition of the liquid agar; absent from solid particles.

Then, 250 mL of the broth was poured in a 500 mL conical flask and autoclaved for 20 minutes at 121 ºC.

3.2.2.3 Inoculum Preparation

The *B.cereus* culture that maintained at 4 ºC was used to inoculate onto the agar plates. The ampoule containing the culture was brought under the laminar flow and the it was shaked from side to side to suspend the organism and to avoid moisten cap on the tube. The cap of the ampoule was flamed and removed but without putting it on the table. The neck of it was also flamed. Then, the inoculating loop was flamed until red hot and the handle was also flamed slightly. The inoculating loop was cooled down by putting the loop on the agar. After it was cooled (about 1-3 seconds), *B.cereus* culture was activated by taking one loopful of it without touching the side of the ampoule and streaked it onto the agar. The ways of streaking for regeneration or subculturing was shown in Figure 3.1.

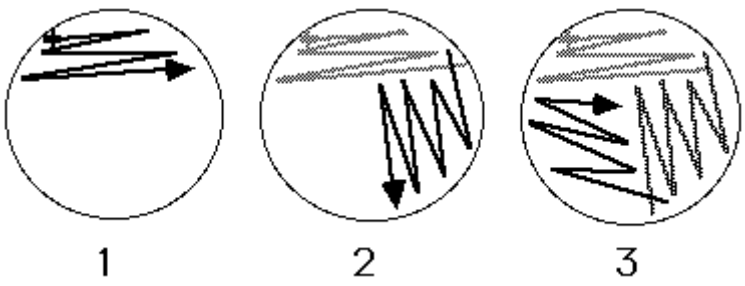

Figure 3.1 Streaking method for subculture and activation (C. Helms, 1990)

Then, the streaked agar are sealed using parafilms and stored in an incubator at 30 ºC in inverted manner. This was to ensure no vapourisation occured.

After the culture was grown for 24 hours at 30 ºC, a colony of the bacteria was taken to be suspended in the broth inside the 500 mL conical flask broth. Then, it was incubated for 2 days at 30 ºC at 250 rpm. This was then called as liquid suspension and transfered into 90 mL of nutrient broth for activation process at 30 ºC at 250 rpm for 30 hours. Then, the process was proceeded with the inoculum process with the same steps as the activation process for 18 hours.

3.3 Growth Curve for *B.cereus*

A colony from the agar plate was inoculated into the standard medium; 225 mL distilled water, 0.5 g yeast extract, 2.5 g peptone and 0.25 g $MgSO_4.7H_20$ in a 500 mL shake flask. Then the bacteria was fermented in the medium at 30 ºC and 250 rpm

The sample will be taken for every 3 hours until hour 30. Then, OD was observed by using UV-Vwas at 650 nm (Berger *et al*., 2003). After that, the sample was observed for every 6 hours until it reached death phase (approximately hour 60).

3.4 Delignification Process

After the inoculum was ready, delignification of rice straw can be executed. Thwas was done by fermentation process.

3.4.1 Fermentation

Four 500 mL conical flask was prepared. Each contains 250 mL of the medium broth of 2.5 g of peptone, 0.5 g of yeast extract and 0.25 g of $MgSO_4.7H_2O$ and sterilized for 20 minutes at 121 ºC.

After that, 10 g of rice straw and 10 % of inoculums were added to three of the flasks. Whreas one flask contained 10 g of rice straw without the inoculum and indicated as Control. Then, it was incubated in an incubator shaker at 30 ˚C and 250 rpm for 2 days.

3.4.2 Method of Analysis

When the period of fermentation was completed , the samples were harvested and the lignin content was analyzed. The method used was Klason's Method.

3.4.2.1 Dried Rice Straw

The sample was dried in oven at 100 ºC for an hour. After that, 1 g of the sample was taken out to be analyzed. The sample was placed in a 100 mL beaker.

3.4.2.2 Addition of Sulphuric Acid (H_2SO_4)

Twenty mililiters of 72 % purity of H_2SO_4 was added to the sample and let it be at room temperature. (25 ºC) for 2 hours.

3.4.2.3 Addition of Distilled Water

Five hundred mililiters of disstilled water was added to the sample and heated at boiling temperature (100 ºC) for 2 hours and 700 rpm in a shaking water bath.

3.4.2.4 Filtration

The samples was filtered and the acid left in the sample was washed with distilled water by checking it using pH meter. Then, the sample was dried again in the oven at 105 ºC for 1 hour. After that, the sample was placed in the desiccator to make sure that there are no mowasture left while waiting for the sample to cool down for 2 days.

Finally, the sample was weight and measured using the equation to determine the lignin degradation:

$$\% \text{ of Lignin degradation} = \frac{x_1 - x_2}{x_1} \times 100\%$$

Where, x_1 = % of lignin content without inoculum

x_2 = % of lignin content with inoculum

3.5 Production of PHB

After delignification process, further fermentation was required to produce PHB.

3.5.1 Medium Preparation

4 conical flasks were prepared. But each containing different types of medium. Flask 1 contained 225 mL distilled water, 0.5 g yeast extract and 2.5 g peptone (Medium A). Flask 2 cointained 225 mL distilled water, 0.5 g yeast extract and 0.25 g $MgSO_4.7H_2O$ (Medium B). Flask 3 cointaned 225 mL distilled water 2.5 g peptone and 0.25 g $MgSO_4.7H_2O$ (Medium C). Lastly, flask 4 contained 225 mL distilled water, 0.5 g yeast extract, 2.5 g peptone and 0.25 g $MgSO_4.7H_2O$ (Medium D). Then, all are sterilized at 121 ºC for 20 minutes.

3.5.2 Fermentation

Five grams substrate that had been delignified were added into each flask. Then, each were fermented in a shaker incubator at 30 ºC for 2 days and 250 rpm.

3.6 Analysis of PHB Yield

When the hervest time was reached, the yield of PHB was analysed. 10 mL of sample was centrifuged at 5000 rpm for 12 minutes at 4 ºC. Then, the pellet was resuspended with 10 mL 0.625 % sodium chloride (NaCl) by shaking and using vortex machine. The sample was centrifuged again at 5000 rpm for 12 minutes at 4 ºC. After that, 10 mL of 100 μM hyrogen peroxide (H_2O_2) was poured into the centrifuge tubes and let to be in the shaking water bath at 30 ºC for 4 hours. Then, the sample was centrifuged again at 5000 rpm for 12 minutes at 4 ºC. Following after that, 10 mL of chloroform was added to extract the PHB. Then, the solution was poured into a petri dish and the sample was dried in an oven at 60 ºC for 1 hour. After that, 5 mL hydrochloric acid (HCl) was added into the petri dish and transfered into a glass test tube where it will be converted into crotonic acid. The test tubes were then put into boiling water bath at 100 ºC for 10 minutes. After it had cooled

down, the OD were checked at 238 nm by using UV-Vwas spectrophotometer. Then, the OD are compared to the standard PHB concentration curve to obtain the concentration of the PHB for determination of the yield.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter includes results of the growth curve for *B.cereus*, analysis of delignification of rice straw by *B.cereus* and analysis of PHB yield by *B.cereus*.

4.2 Growth Curve of *B.cereus*

The determination the growth curve of *B.cereus* was taking approximately 60 hours or 15 sampling. The bacteria was activated in liquid suspension for 30 hours and inoculated for another 18 hours at 30 ºC and 250 rpm. After that, the bacteria was cultured in 250 mL dwastilled water, 0.5 g yeast extract, 2.5 g peptone and 0.25 g $MgSO_4.7H_20$ in a 500 mL shake flask. Then the bacteria was fermented in the medium at 30 ºC and 250 rpm. The data is in Table 4.1 whereas the growth curve is in Figure 4.1:

Table 4.1 : Growth curve of *B.cereus* sampling data

Sampling no.	Time(hr)	Blank	Medium
1	3	0	1.475
2	6	0	1.704
3	9	0	2.523
4	12	0	2.125
5	15	0	2.409
6	18	0	2.699
7	21	0	2.409
8	24	0	2.671
9	27	0	2.568
10	30	0	2.579
11	36	0	2.584
12	42	0	2.638
13	48	0	2.745
14	54	0	2.74
15	60	0	2.342

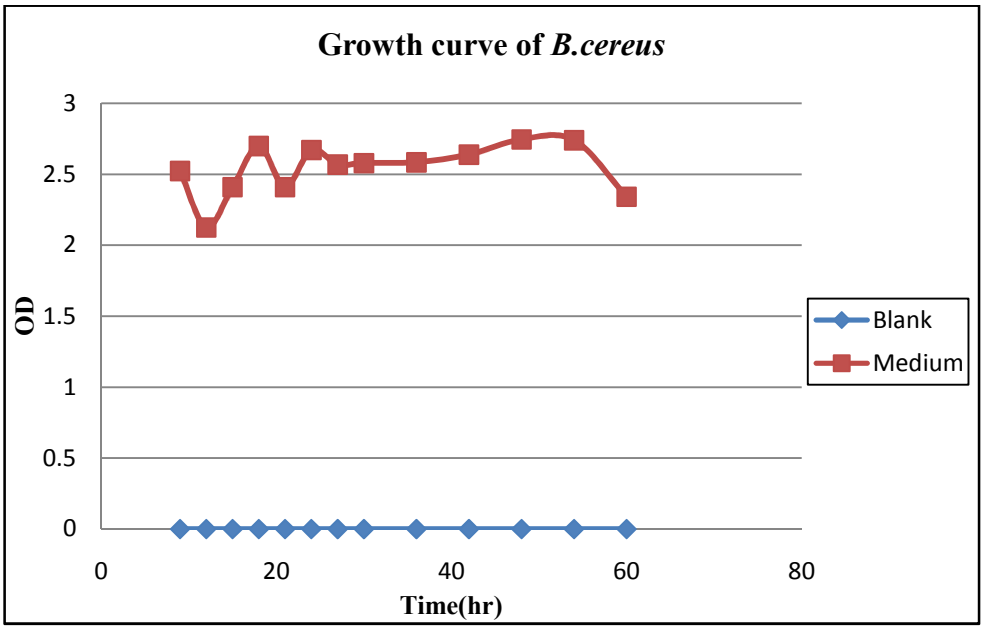

Figure 4.1 Growth curve of *B.cereus*

From the result obtained, from hour 3 to 24, the growth curve is not stable and fluctuating. This is due to the motility of *B.cereus*. The spores lose their motility during early stages of sporulation (Granum, 2007). The fluctuation can also be caused by the errors occured while preparing the medium and it can be improved by taking the sample more frequently such as sampling every hour.

Based on the observation from Figure 4.1, the graph is divided into three phases which are lag, log or exponential, stationary and death phase. The lag phase starts hour 24 to 36. The number of cells changes very little because the cells do not immediately reproduce in a new medium. During this time, it is not dormant. *B.cereus*'s population is undergoing a period of intense metabolic activity, especially deoxyribonucleic acid (DNA) and enzyme synthesis (Tortora *et al*., 2002). *B.cereus* is also given time to adapt with the new environment after become inactive for a long period time. Therefore, when running the fermentation process, the *B.cereus* is already adapting into the new environment and became active.

The exponential phase starts from 27^{th} hour to 48^{th} hours. *B.cereus* devides to form two cells, and each divides to form another two cells for a brief extended period. This depends on the available resources and other factors. For *B.cereus,* it fully utilizes the availability of nutrients At this state, it is the healthiest (Madigan *et al*, 2006). The rate of growth is constant during the exponential phase because the microorganisms are dividing at regular intervals.

Then, it entered stationary phase at hour 54. This is occured because the nutrients are depleted and wastes accumulate thus the rate of reproduction decreases and the the population growth decreases. Eventually, the number of dying *B.cereus* equals the number of it being produced. This resulted in the stationary of the population size. At this phase, its metabolic rate of the surviving bacteria declines (Bauman, 2006). Other theories revealed that the cause of stationary phase may be of two factors; a balance between cell growth 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. When the availability of the nutrients is depleted, population growth rate

will dwindle. In this case, oxygen limitation is also one of the factors due to its aerobic metabolism. Oxygen is poorly soluble and may be depleted rapidly that only the surface of the culture receives oxygen that is adequate enough for growth. Third factor is the accumulation of toxic waste products that can inhibit the growth process of the bacteria.

After that, the graph was entering the death phase after 60^{th} hours. At this phase, the number of deaths exceeds the number of new bacteria formed (Tortora *et al*., 2002). This is due to detrimental environment changes like nutrient deprivation and the buildup of toxic wastes.

To obtain the growth curve, the value of the optical density (OD) were taken by every three hours for the first of 30 hours. Then the OD value was taken for every six hours until the OD value is decreasing. The OD value were obtained by using UV-Vis spectrophotometer at 650 nm (Berger *et al*., 2003).

4.3 Analysis of Lignin Content

Based on the growth curve, *B.cereus* was cultivated from the inoculum at hour 48 for the fermentation. In the fermentation, the mixture of the standard liquid medium, bacteria and rice straw were put in 3 conical flask and left for 2 days. Another flask that had no inoculum acted as a Control. After 48 hours, the rice straw was filtered from the medium and analysis was carried on by using Klason's method.

There are 3 steps; drying, boiling and filtering methods. For the drying step, the samples were dried at 105°C for 1 hour. The objective of this step was to get a constant dry weight. Then, 1 g of the sample was mix with 20 mL of 72% purity of acid sulfuric (H_2SO_4) for 2 hours. H_2SO_4 was used to dissolve polysaccharides, proteins and other polymer components including lignin.

To ensure that all the components were fully dissolves, the boiling step was conducted. The solution was mixed with 500 mL of distilled water and left in shaking water bath at 100°C and 70 rpm of agitation speed for 2 hours.

After that, the last step was proceeded; that was the filtering step. The samples were filtered and rinse with distilled water to wash away H_2SO_4. The filtrate from the filter process was dried at 105°C for 1 hour to get constant dry weight. Finally, the sample was left in the desicator for 2 days. The objective was to ensure the sample was fully dried and cooled to room temperature.

After that, the final weight from the sample indicate the amount of lignin lost from the fermentation of rice straw. The difference amount of lignin between the sample without *B.cereus* and the sample with the *B.cereus* indicated the amount of lignin degradation.

The equation used to determine the lignin content (%) in each sample was:

$$\text{Lignin content (\%)} = \frac{Wlignin}{Wfiber} \times 100\%$$

(Garcia *et al*., 2008)

Where, W_{lignin} = oven-dry weight of insoluble lignin

W_{fiber} = oven-dry weight of wood fibers

Percent of lignin degradation was calculated as below:

$$\% \text{ of Lignin degradation} = \frac{x_1 - x_2}{x_1} \times 100\%$$

Where, x_1 = % of lignin content without inoculum

x_2 = % of lignin content with inoculum

Table 4.2 : Delignification data

Sample	Initial mass rice straw (g)	Final mass rice straw (g)	Lignin content (%)	Lignin degradation (%)
1	1.0014	0.035	3.49	95
2	1.0116	0.0016	0.16	98
3	1.0078	0.046	4.56	43
4 (control)	1.0046	0.0822	8.18	-

Lignin content for each sample was 3.49 %, 0.16 %, and 4.56 %. Whereas the Control was 8.18 %. This shows that there was 8.18 % lignin available in 1 g of rice straw. After fermentation process, the lignin content in the sample must be much more reduced than in the Control because there was no bacteria to delignify the lignin in the rice straw.

The percentage of lignin degradation for the three samples were 95 %, 98 % and 43 % respectively. The average lignin degradation was 78.67 %. This percent of lignin degradation shows the amount of which the bacteria was able to degrade it. For Sample 1, there was 95 % of lignin degradation based on the lignin content of

Control. Whereas, bacteria in Sample 2 was able to degrade 98 % of lignin based on Control and finally, in Sample 3, 43 % of lignin from the 8 % of Control was able to be degraded. Sample 3 has the lowest lignin degradation due to error while inoculating the bacteria by less suspending the the bacteria when introduced into the sample. Thus, there was less bacteria to degrade the lignin compared to other samples.

These degradations of lignin occured because of the enzymatic process by *B.cereus.* The enzymes produced are able to oxidize the aromatic ring of the lignin thus electrophilic substitution reactions can occur (Carraher *et al.,* 2008). The oxidation proces a ruptured of –C-O-C- bonds of the lignin (Gnanou *et al.,* 2008).

4.4 Analysis of PHB Yield

4.4.1 PHB Standard Curve

To analysis the PHB yield, the concentration of PHB respective to its OD was needed. This was determined by first doing the standard curve for OD vs PHB concentration as in Table 4.3 as follow:

Table 4.3 : Data for PHB standard curve

Sample (mg/ml)	1st reading	2nd reading	3rd reading	Average (OD)
0	0.000	0.000	0.000	0.000
20	0.299	0.293	0.283	0.292
30	0.421	0.423	0.425	0.423
40	0.632	0.638	0.641	0.637
50	0.763	0.767	0.765	0.765
60	0.860	0.870	0.869	0.879
70	1.060	1.058	1.068	1.064
80	1.217	1.209	1.214	1.218
90	1.302	1.306	1.303	1.304
100	1.509	1.521	1.519	1.520

The graph was plotted as in Figure 4.2 below.

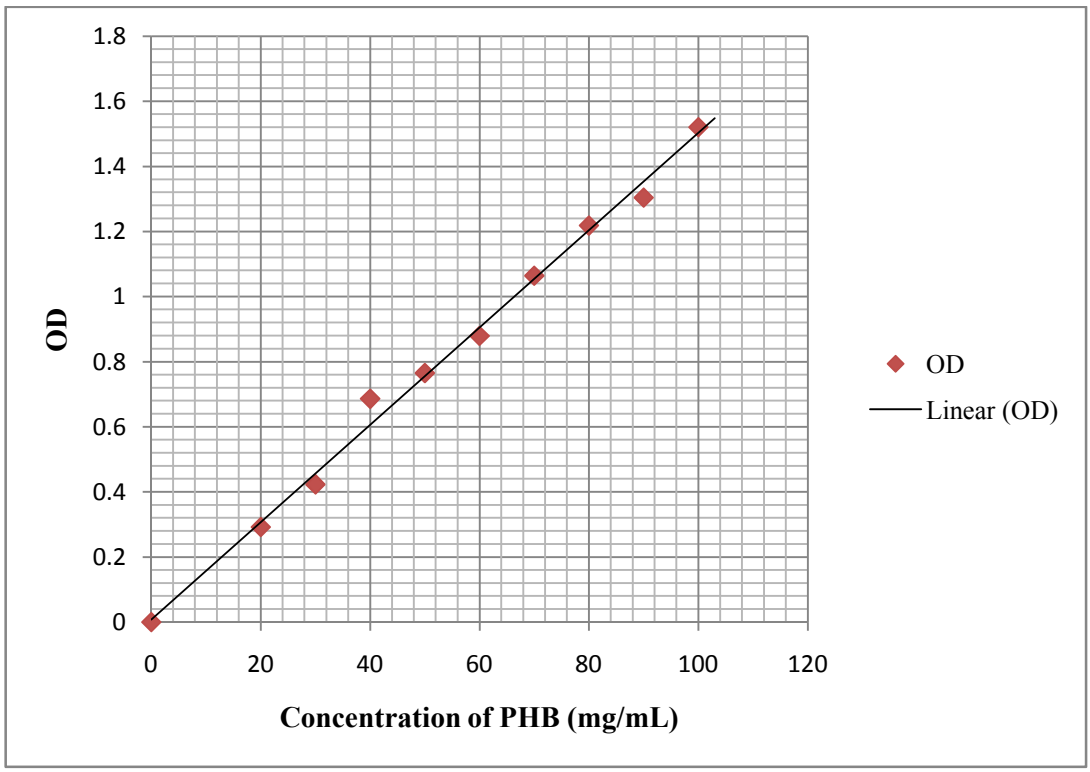

Figure 4.2 Graph for standard PHB curve

The PHB standard curve was used to determine the concentration of PHB when only the OD of the PHB was obtained. When the absorbance was observed at 235 nm, the OD obtained will be matched at the graph plotted to know the concentration. After concentration was obtained, only then the yield of PHB from *B.cereus* is known.

4.4.2 Analysis of PHB Yield

The analysis is using the UV-Vis method to observe the absorbance. Yield was calculated by cell dry weight divided by PHB mass, $Y_{X/P}$. The results were as in Table 4.4 below:

Table 4.4 : Data of PHB yield

Medium	OD	PHB Conc. (mg/mL)	Cell dry weight (mg)	Yield (%)
Peptone + yeast (Medium A)	0.9743	64.0	15.4	2.40
$MgSO_47H_2O$ + yeast (Medium B)	0.758	50.0	13.1	2.62
Peptone +$MgSO_47H_2O$ (Medium C)	1.3857	91.2	18.5	2.03
Peptone +$MgSO_47H_2O$ + yeast (Medium D)	0.848	56.0	14.0	2.50

From the results obtained, rice straw can be used as substrate. The rice straw contained cellulose that had been used as carbon source. To produce PHB, the cellulose must be degraded into monomers first. This was due to the biochemical pathways leading to PHB production. In estimating the yields, a special emphasis was made on recycling (or regeneration) of $NADP^+$ which was the co-substrate of acetoacetyl-CoA reductase, one of three key enzymes involved in the biosynthesis of PHB. As a $NADP^+$-regenerating enzyme, glucose-6-phosphate dehydrogenase or isocitrate dehydrogenase was conceived (Yamane, 1992). Thus this proved that monomer must involved to synthesis PHB. For further understanding, Figure 4.3 illustrated how PHB was synthesized by metabolism of bacteria: Whereas the pathway to produce PHB was as in Figure 4.4. It can also be deduced that, the low yield was due to the genetic of *B.cereus*' metabolism. Between PHB pathway and citric acid cycle, its metabolism was more preferable towards citric acid cycle.

For cellulose hydrolysis, the bacteria needed at least, three different enzymes for the complete hydrolysis of the crystalline. Those were endo-ß-glucanase, exo-ß-glucanase and cellobiase. This was due to the straight chain of B-1,4-linked glucose units without any side chains. Thus, extensive hydrogen bonding between the cellulose molecules formed the crystalline structure. (Kim, 2008).

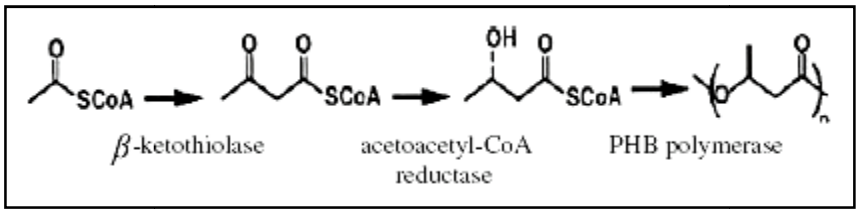

Figure 4.3 Polyhydroxybutyrate (PHB) biosynthetic pathway. PHB is synthesized by the sequential action of b-ketothiolase (phbA), acetoacetyl-CoA reductase (phbB), and PHB polymerase (phbC) in a three-step pathway (Madison and Huisman 1992)

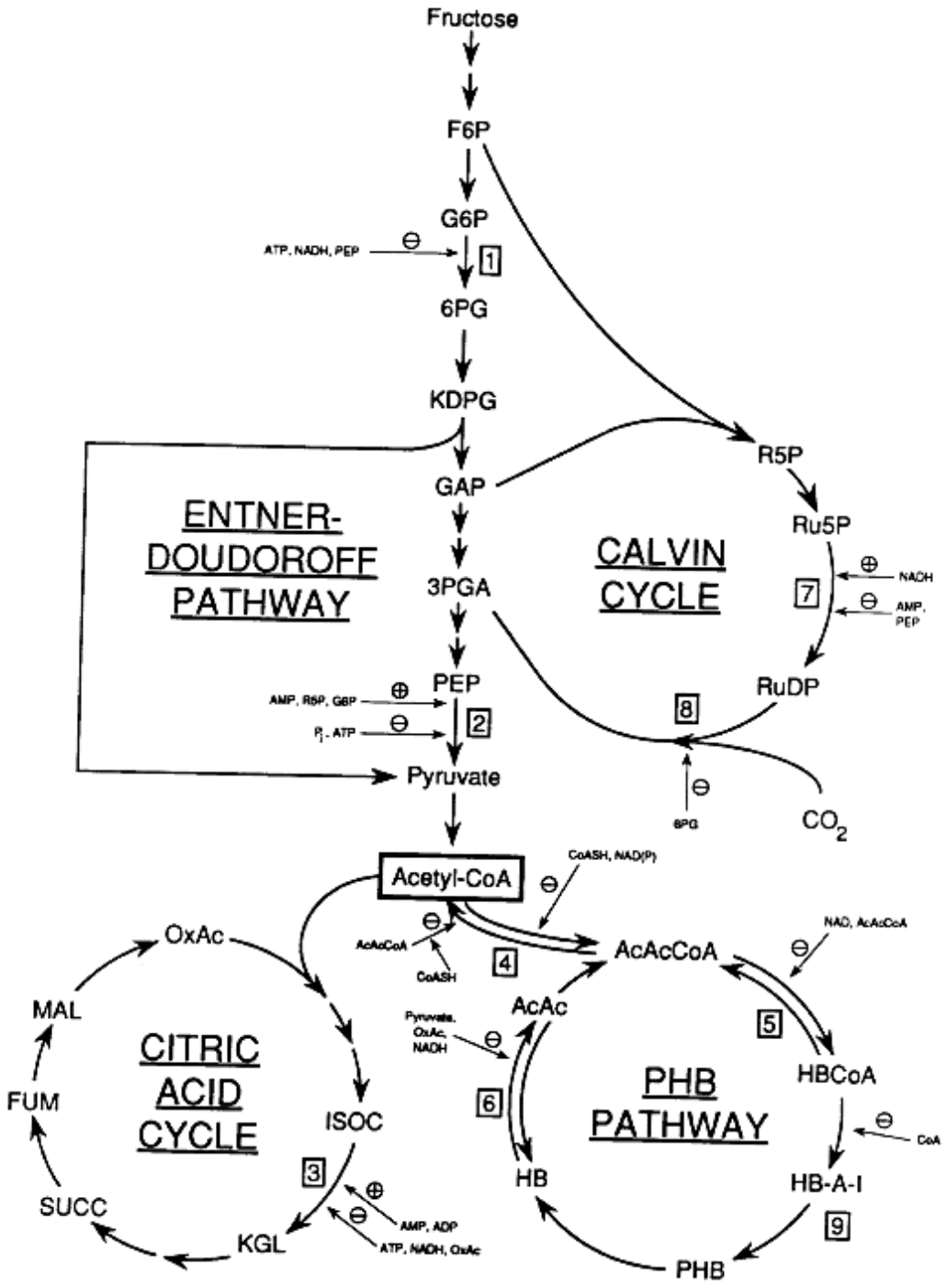

Figure 4.4 Metabolic pathway to produce PHB (Guske, 1990)

The yield of PHB was observed by using the PHB standard curve. From the result obtained, the highest PHB yield is produced in Medium B that was consisted of $MgSO_4.7H_2O$ and yeast that yield 2.62 %. Yeast is the source of vitamin and it is had the least effect on cellular growth and no effect on PHB concentration and

content (Sangkharak, 2008). Thus it does not effect the PHB production at all, whether it is available or not. The nutrient that was missing is peptone. Peptone is a source of nitrogen. In a cell, 12 % of the cell dry weight is nitrogen and important in proteins, nucleic acids and several other cell constituents (Madigan *et al*., 2006). According to Kanokphorn Sangkharak *et al* (2008), the highest yield of PHB achieved when carbon and energy sources are in excess but lack in oxygen, nitrogen or phosphorus.

The second highest yield is PHB produced in Medium D that contained peptone, $MgSO_47H_2O$ and yeast of 2.50 %. This was due to the peptone content in the medium that enhances sporulation (Adams *et al*., 2008). Thus, there is enough *B.cereus* to metabolize the cellulose to yield PHB. Magnesium is important to stabilize ribosomes, membranes, nucleic acids and required for the activity of many enzymes (Madigan *et al.,* 2006). It also acts as buffer and supplied as $MgSO_47H_2O$. Addition of it is important for cell growth and loss of buffer capacity led to high pH that might inhibit the growth level (Sangkharak *et al*., 2008).

Whereas, Medium A; peptone and yeast had 2.40 % PHB yield. Lastly, Medium C that was contained peptone and $MgSO_47H_2O$ with 2.03 % of PHB yield. Both medium contained peptone. Thus this proved that due to higher concentrations of nitrogen sources, cell growth and PHB concentration increased but PHB content decreased. In contrast, when it is low in nitrogen concentration, PHB content increased. Yeast is the source of vitamin and it had the least effect on cellular growth and no effect on PHB concentration and content (Sangkharak, 2008).

Theoritically, studies that had been done by Vijayendra *et* al (2007), many bacteria synthesize PHAs (this includes PHB) intracellularly under certain nutrient depleted conditions, presence of excess carbon and less nitrogen source, as PHA formation is favoured by conditions that led to high NADPH, high acetyl CoA and low free coenzyme A concentrations inside the cells. The β-ketothiolase, a key enzyme in PHA synthesis, is completely inhibited by high concentrations of COASH, when grown in nutrient rich medium with out any limitation of carbon and nitrogen. Under unbalanced growth conditions, acetyl-CoA concentrations remain

high but free CoA is low, thus avoid inhibition of β-ketothiolase activity. So, from the actual results obtain, the medium that contains all the nutrient is supposedly to have the lowest PHB yield. The result is was obtained by the error while doing the medium, culturing or OD reading.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this paper, there are 2 main objectives. Those are to delignify rice straw and to produce PHB both by using *Bacillus cereus*. Delignification is crutial because its chemical durability makes it indigestible to organisms because of its bonding to cellulose and protein material. This lignin sheet acts as a barrier towards the outside elements (Carraher, 2008). From the result, lignin content for each sample is 3.49 %, 0.16 %, and 4.56 % and the Control is 8.18 %. The percentage of lignin degradations for the three samples is 95 %, 98 % and 43 % respectively. The average lignin degradation is 78.67 %. This percent of lignin degradation shows the amount of which the bacteria is able to degrade it. After delignification, the bacteria can act on the cellulose content to produce PHB. Enzymes that can utilize cellulose are C_1, Glucanase or Cx and β-glucosidase. (Rajvaidya *et al*., 2006). Then, *Bacillus cereus* can synthesize PHB by utilizing acetyl-CoA by the sequencial action of 3 enzymes; B-ketothiolase (phbA), acetyl-CoA reductase (phbB) and PHB polymerase (phbC) (Redd *et al.,* 2003). From the result obtained, the highest PHB yield is produced in the medium consists of $MgSO_4.7H_2O$ and yeast that yield 2.62 %, the second highest yield is PHB produced in the peptone, $MgSO_47H_2O$ and yeast medium of 2.50 %. Whereas, peptone and yeast contained medium has 2.40 % PHB yield. Lastly, peptone and $MgSO_47H_2O$ with 2.03 % of PHB yield. Thus, from the results obtained, objectives of this paper are achieved.

5.2 Recommendation

For the delignification process, instead of using gauze cloth, it is recommended to use filter or Whatman glass microfiber. This is to ensure that no rice straw will be wasted away in the drains. Big pores in the gauze cloth made the filtering faster, but the result will not be accurate. Also, when using the filter, to hasten up the process, it is recommended to use vacuum filter. It is recommended as well to degrade it in anaerobic condition, temperature of 37 ºC (as *B.cereus* is mesophile), and availability of nitrogen (Rajvaidya *et al*., 2006).

For the PHB production, it is recommended to hydrolyse the cellulose and hemicellulose into their monomers, glucose and xylose before fermentation process. This is because bacteria are easier to consume simple sugar than polysaccharides thus better production of PHB (Yamane, 1992). It is also advisable to do the experiment in an anaerob condition because when the bacteria is restricted in nutrient such as oxygen, the PHB production will increase. To optimize it, the parameters should also be variables.

To analyze the PHB, it is most accurate to use HPLC instead of UV-Vis. If there is no column available, make it so by ordering it beforehand 6 months ahead the experimentvor by doing the analysis at a place that accommodate HPLC along with the column. In addition, because of the PHB has converted into crotonic acid, a form of liquid form solid, it is advisable to use filter paper and filter pump to make the result more by decreasing the contamination by other by-products or substances.

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APPENDIX

1. Delignification Process

Mass of dried rice straw

Sample	Mass of rice straw after dried (g)	Mass of rice straw taken to be fermented (g)	Mass of rice straw after fermentation (g)
1	10.0428	1.0014	0.035
2	10.0372	1.0116	0.0016
3	10.0253	1.0078	0.046
4 (Control)	10.0172	1.0046	0.082

2. Production of PHB

Analysis of PHB by using UV-Vis

Sample	Blank (OD)	10x dilution (OD)			Average (OD)
Medium A	0.000	0.975	0.976	0.972	0.97433
Medium B	0.000	0.758	0.758	0.758	0.758
Medium C	0.000	1.374	1.381	1.402	1.3857
Medium D	0.000	0.848	0.848	0.848	0.848

Result of PHB production

Sample	Dry cell weight (mg)	OD	PHB Concentration (mg/mL)	Yield (%)
Medium A	5.4	0.9743	64.0	2.40
Medium B	3.1	0.758	50.0	2.62
Medium C	8.5	1.3857	9.2	2.03
Medium D	4.0	0.848	56.0	2.50

3. Calculations

Lignin content

Sample 1

$$\frac{0.0035}{1.0014}$$

$$= 0.349\ \%$$

Sample 2

$$\frac{0.0016}{1.0078}$$

$$= 0.1582\ \%$$

Sample 3

$$\frac{0.046}{1.0078}$$

$$= 4.56\ \%$$

Sample 4 (Control)

$$\frac{0.0822}{1.0046}$$

= 8.00 %

Stock Solution NaCl (from solid to liquid)

MW NaCl = 58.44 g/mol

Volume to dilute = 50 mL distilled water (since volumetric flask available was 50 mL)

$$M_{solid} = \frac{mass}{MW \cdot V}$$

Mass NaCl = (0.6 mol/L) (58.44 g/mol) (50 mL) (1 L/ 1000 mL)

= 1.82625 g NaCl in 50 mL dilution

Preparation of 100 µM hydrogen peroxide

Purity = 30 % Density = 1.11 g/cm^3

MW= 34.02 V to be diluted = 50 m:L

$$\text{Molarity } (M_1) = \frac{(1.11 \text{ g/cm}^3)\ (0.3)\ (1000)}{34.02 \text{ mol/g}}$$

M_1 = 9.788 mol/mL

$M_1V_1 = M_2V_2$

(9.788) (V_1) = (1000 µmol/mL)(50 mL)

V_1 = 5.10829 µL was pipetted in 50 mL dilution