DELIGNIFICATION OF BANANA STEM WASTE IN ANAEROBIC CONDITIONS BY USING *Bacillus cereus*

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DELIGNIFICATION OF BANANA STEM WASTE IN ANAEROBIC CONDITIONS BY USING *Bacillus cereus*

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

Faculty of Chemical and Natural Resources Engineering
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APRIL 2009

I declare that this thesis entitled "*Delignification of Banana Stem Waste in Anaerobic Conditions by Using Bacillus cereus*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not currently submitted in candidature of any other degree.

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To my dearly loved mother, Robitah binti Hijaz,

father, Baharuddin bin Abd Rahim,

and sisters

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ABSTRACT

Today, the productions of biodegradable plastics are crucial due to the environment problems that keep escalating caused by conventional plastics. Many efforts have been done to reduce the cost of biodegradable plastic including by using waste as raw material. Production of biodegradable plastics by microorganism needs carbon sources. Several ranges of microorganisms have been tested for their ability to degrade lignin in order to obtain cellulose content to be used as carbon source. In this study, the submerged fermentation in anaerobic conditions of banana stem waste using *Bacillus cereus* and the capability of *Bacillus cereus* to delignify banana stem waste in anaerobic conditions are investigated. This study was divided into three parts which are the revival of *Bacillus cereus*, growth curve determination of *Bacillus cereus*, and delignification fermentation and analysis of *Bacillus cereus*. Fermentation was carried out anaerobically in batch mode at 30 ^{o}C and 37 ^{o}C. The analysis was done in accordance to Klason method. Results showed that *Bacillus cereus* was able to survive for 48 hours in anaerobic conditions and it is able to degrade lignin from banana stem waste. The optimum temperature for *Bacillus cereus* to degrade lignin was at 30 ^{o}C with average percentage of lignin degradation of 13.33 %.

ABSTRAK

Dewasa ini, penghasilan plastik terbiodegradasikan menjadi amat penting memandangkan masalah alam sekitar yang kian meningkat disebabkan oleh plastik biasa. Banyak usaha telah dilakukan bagi mengurangkan kos penghasilan plastik terbiodegradasikan ini termasuk penggunaan sisa sebagai bahan mentah. Penghasilan plastik terbiodegradasikan ini memerlukan sumber karbon sebagai nutrien bagi mikroorganisma. Beberapa jenis mikroorganisma telah diuji kebolehannya untuk mengurangkan lignin dalam sisa tumbuhan bagi mendapatkan kandungan selulosa di dalamnya untuk digunakan sebagai sumber karbon. Dalam kajian ini, kaedah fermentasi terendam batang pisang dalam keadaan anaerobik menggunakan *Bacillus cereus* dan kebolehan *Bacillus cereus* untuk mengurangkan lignin dalam batang pisang telah dikaji. Kajian ini dibahagikan kepada tiga bahagian iaitu menghidupkan semula *Bacillus cereus*, penentuan graf pertumbuhan bagi *Bacillus cereus*, dan proses fermentasi dan analisis bagi pengurangan lignin oleh *Bacillus cereus*. Proses fermentasi telah dijalankan secara anaerobik pada suhu 30 ^{o}C and 37 ^{o}C. Manakala proses analisis telah dijalankan mengikut kaedah Klason. Hasil kajian menunjukkan bahawa *Bacillus cereus* berupaya untuk hidup selama 48 jam dalam keadaan anaerobik. Ia juga terbukti boleh mngurangkan lignin dalam batang pisang. Suhu optimum bagi *Bacillus cereus* untuk mengurangkan lignin dalam keadaan anaerobik ialah pada 30 ^{o}C dengan peratusan purata sebanyak 13.33 %.

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LIST OF SYMBOLS/ABBREVIATIONS

%	-	Percentage
^{o}C	-	Degree Celcius
g	-	Gram
kg	-	Kilogram
L	-	Liter
$MgSO_4.7H_2O$	-	Hydrated magnesium sulphate
mL	-	Mililiter
mm	-	Milimeter
rpm	-	Rotation per minute
sp.	-	Species
v/v	-	Volume per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

In the recent years, lack of degradability, the closing of landfill sites, and the growing of water and land pollution problems have led to concern about plastics. Conventional plastics that are synthetically derived from petroleum are not readily biodegradable (Huang *et al*., 1991; Young, 1981) and are considered as environmentally harmful wastes (Hong Kong Environmental Protection Department, 1994). Plastic materials account for about 20% by volume of municipal solid wastes and reduce the capacity of precious landfill sites (Lee and Yu, 1997). This is why there are growing interests in the production of bioplastics. Environmentally friendly biodegradable plastic, or simply bioplastic, have been developed either by incorporating natural polymers into conventional plastic formulations, by chemical synthesis, or by microbial fermentations (Chua *et al*., 1995; Chang, 1994). Despite receiving much attention as a biodegradable substitute for conventional non-biodegradable plastics, the commercial use of bioplastics remains limited because of its high production cost (Jung *et al*., 2005).

A family of more than 40 poly-hydroxy alkanoates (PHAs) and their co-polymeric derivatives are considered as very attractive materials in producing bioplastic due to their complete biodegradability (Kumagai, 1992). They are polymers produced by microorganisms as inclusion bodies, susceptible to biodegradation into carbon dioxide and water (Kapritchkoff *et al*., 2006). Poly-β-hydroxybutyric acid (PHB) is the

most extensively studied among the poly-hydroxy alkanoates in nature in the presence of excess carbon by bacteria as storage granules providing food, energy and reducing power (Kapritchkoff *et al*., 2006; Salehizadeh and Van Loosdrecht, 2004; Pfeffer, 1992). PHB has properties similar to petroleum derived synthetic plastics like polypropylene (PP) and is completely biodegradable in the environment (Khardenavis *et al*., 2007).

In 1997, Chua et al. stated that the cost of producing PHB bioplastic is ten times higher than that of conventional plastics. Later in 2004, Serafim *et al*. reported that the cost has reduced to nine times higher than the cost of conventional plastics. Up to this day, there have been, and still are, many efforts done by scientists and researchers in order to optimize the production of PHB and reducing the cost. For instance the development of better bacterial strains, and efficient fermentation and recovery systems (Choi *et al*., 1998; Wang and Lee, 1997; Lee, 1996). On the other hand, excess activated sludge from a wastewater treatment plant is used as a source of PHB, and renewable carbon resources derived from agriculture or industrial wastes are used as substrate for PHB accumulation (Suresh Kumar *et al*., 2004; Chua and Yu, 1999; Braunegg *et al*., 1978). By these approaches, the cost on biomass generation can be reduced, apart from volume reduction of waste activated sludge by extracting PHB (Khardenavis *et al*., 2007).

Researches have been done in accordance to selection of efficient PHB producers. Among the PHB producers are *Alcaligenes* sp., *Azotobacter* sp., *Bacillus* sp., *Nocardia* sp., *Pseudomonas* sp., and *Rhizobium* sp. These microorganisms need carbon as their energy source. Plant waste fibers such as banana stem waste have high content of cellulose, which can be used as carbon sources for microorganisms. However, in order to obtain the cellulose, the banana stem waste need to be delignified. In previous studies, several microorganisms have been tested for their ability to degrade lignin on different raw materials. Nonetheless, the aim of this study is to assess the cellulose recovery potential through lignin degradation by bacteria *Bacillus cereus* using banana stem as feedstock

1.2 Objectives of Study

Based on the background of this study, the objectives of this study are listed as following:

i. To study the submerged fermentation in anaerobic conditions of banana stem waste using *Bacillus cereus*.
ii. To study the ability of *Bacillus cereus* in delignifying banana stem waste in anaerobic conditions.

1.3 Scope of Study

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

i. Submerged fermentation in anaerobic conditions as the incubation condition for lignin degradation process of banana stem waste using *Bacillus cereus*.
ii. Test the efficiency of lignin degradation of banana stem waste in anaerobic conditions by *Bacillus cereus*.

1.4 Problem Statement

Plant waste fibres can be explained as lignocellulosics, which means, resources consist primarily of cellulose, hemicellulose, and lignin. The production of PHB utilizes cellulose. The presence of lignin slows down the microbial attack for the degradation of cellulose. Thus complete degradation does not occur and most of the cellulose remains undigested. To enhance the bioplastic production from such substrate it is necessary to

make cellulose components free from lignin. Biological delignification is a promising process for the preparation of suitable substrate for PHB bioplastic generation. This is where the issue arises. In order to achieve satisfactory delignification, suitable microorganisms have to be used. In the earlier researches, many microorganisms such as actinomycetes, fungi and bacteria have been tested for the ability to delignify lignocellulosics. By using banana stem waste as lignocellulic source; this study aims to discover the ability of *Bacillus cereus* to biologically degrade lignin in banana stem in order to attain the cellulose for PHB production.

1.5 Rationale and Significance

The degradation of lignin in banana stem waste by using bacteria *Bacillus cereus* brings excellent justifications. Firstly, lignin needs to be degraded in order to obtain the cellulose which can then be further utilized as a substrate in producing PHB bioplastics. Secondly, as the raw material to be used is waste, it is substantially cheap and widely available here in Malaysia since there are a lot of banana plantations in this country. Moreover, banana stem is known to contain a high content of cellulose. Lastly, if the bacteria strain *Bacillus cereus* is able to degrade lignin, the cost will be significantly fewer than that of using chemicals to degrade lignin, due to the elimination of several steps required to recover cellulose. In addition, microbial degradation by *Bacillus cereus* can reduce the probability of contamination of products (i.e. cellulose) by chemical use, since microbial degradation only involves the activity of enzyme.

CHAPTER 2

LITERATURE REVIEW

2.1 Bioplastic and Biodegradation

Recently the term 'bioplastics' has almost replaced the term 'biodegradable plastics'. Bioplastics are now commonly regarded as to be a form of plastics derived from natural resources such as wood (cellulose), vegetable oils, sugar or starch.

Biodegradation is the process by which organic substances are broken down by living organisms. In relation to bioremediation of plastic materials, biodegradation is a process that describes the mineralization of organic structures by microorganisms. These microorganisms convert the bioplastics into carbon dioxide, methane, water and biomass.

To compare the performance of bioplastic and conventional, non-biodegradable plastic, they certainly do not have the same performance characteristics but are fit-for-purpose in a range of specific applications. To review the applications of bioplastic back in the past few years, they are only said to be applied on medical fields due to its high production cost; while conventional plastics dominate all other sectors and fields. Nevertheless, nowadays the applications of bioplastic include biodegradable plastic shopping bags, compostable waste collection bags and compostable or biomass-based

food trays and food service packaging. Their applications in other sectors are currently under development, for instance in the automotive and electronic sectors.

The degradation of biodegradable plastics is due to cell-mediated phenomena (micro-organisms, enzymes, fungi, bacteria). When the degradation is the result of the action of microorganisms in a material and the material is eventually converted to water, carbon dioxide, methane and biomass, the material is considered biodegradable. On the other hand, compostable plastics are degradable due to biological processes that occur during composting and are converted into carbon dioxide, water, and biomass. For both biodegradable and compostable plastics, there are no toxic side effects like toxic residue for water, soil, plants or living organisms. Currently these plastics are based on renewable resources. Though, not all biodegradable materials are compostable.

As for the degradation of bioplastics, microorganisms such as fungi and bacteria can metabolize biodegradable bioplastics. The polymer becomes their source of food and energy. The microorganisms then transform the biodegradable plastic product into carbon dioxide, water and biomass. A certain level of temperature, heat, water and oxygen is required by active microorganisms such fungi and bacteria for effective biodegradation.

Poly β-hydroxy butyrate (PHB), the most studied biodegradable plastics, is a very common and widespread storage material in many micro-organisms. PHB gathers as energy reserve material in many microorganisms such as *Alcaligenes* sp., *Azotobacter* sp., *Bacillus* sp., *Nocardia* sp., *Pseudomonas* sp., and *Rhizobium* sp. In many cases, these microorganisms need carbon as their energy source. Cellulose can be a great carbon source. In order to provide the carbon sources, approaches have been directed towards the usage of nutrient-rich waste. However, usually pretreatment to waste need to be done in order to gain the cellulose content. The way to pretreat depends on the waste type used.

In the present studies banana stem waste is used due to its high cellulose content and availability. Microorganism used to do pretreatment to waste is *Bacillus cereus*. *Bacillus cereus* is to be tested for its ability to degrade lignin content in banana stem, in order to obtain cellulose content inside it.

2.2 Raw Material for Carbon Source (Banana Stem Waste)

There are various choices of raw materials to be used in the production of biodegradable plastics, namely corn, starch, potatoes, sugarcane, and even the types of renewable raw materials such as biomass fraction present in waste from households, municipal waste, dairy industry, paper mills, forestry, etc. Some specific types of bioplastics can even be produced directly by certain plants. Others need to go through several processes before PHB bioplastic is formed.

In this study, banana stem waste is chosen as raw material to eventually produce PHB. Banana stem is known to contain a high content of cellulose (cellulosic fibre), which can be the substrate for microorganisms producing PHB. Agricultural activity involving banana generates large amounts of residues, because each plant produces only one bunch of bananas. After harvesting the fruits, banana stem (also known as the bare psuedostems) are cut and usually left in the soil plantation to be used as organic material. It has been estimated that for every 60 kg of banana grown, 200 kg of waste stem is thrown away. In Malaysia alone, the area for banana plantation is estimated to be 34, 000 hectars (Abdul Khalil *et al*., 2006). Therefore, by utilizing these wastes, it is hoped to be a way of disposing the waste instead of forgo them. Moreover using banana stem waste can significantly reduce the cost of PHB production as it is cheap and widely available.

2.3 Delignification Process

Plant cell wall material is composed of three important constituents: cellulose, lignin, and hemicellulose. Lignin is a complex polymer of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. This complexity has thus far proven as resistant to detailed biochemical characterization as it is to microbial degradation, which greatly impedes our understanding of its effects.

Nonetheless, some organisms, particularly fungi, have developed the necessary enzymes to break lignin apart. The initial reactions are mediated by extracellular lignin and manganese peroxidases, primarily produced by white-rot fungi (Kirk and Farrell, 1987). Actinomycetes can also decompose lignin, but typically degrade less than 20 percent of the total lignin presents (Crawford, 1986; Basaglia *et al*., 1992).

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).

2.2 Microorganism

2.2.1 Nature of *Bacillus cereus*

Bacillus cereus is a gram positive, rod-shaped bacterium that could grow optimally at a temperature range of 30 – 37 ^{o}C with a 250 rpm shaker speed (Valappil *et al.*, 2007). Even though, some strains can grow up to 55 ^{o}C while others can grow as low as 4 – 5 ^{o}C. Many strains from dairy products are able to grow at low temperatures. The growth of *B. cereus* is best in the presence of oxygen, even so, they grows well anaerobically.

Bacillus cereus is a bacterium that has been known as an agent to food poisoning since 1955 (SCIENCE Magazine, 2004). It is a spore-forming organism that occurs naturally in most food (ESR Ltd., 2001). It causes two types of foodborne illnesses- emetic illness and a diarrhoeal illness. The scientific classification of *Bacillus cereus* is shown in Table 2.1.

Table 2.1: Scientific classification of *Bacillus cereus* (Frankland & Frankland 1887)

Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species	*cereus*
Binomial name	*Bacillus cereus* (or *B. cereus*)

Bacillus cereus is a newly characterized strain in the PHB-producing area thus less information regarding its ability to degrade lignin is available elsewhere. There is a research done by Valappil *et al.* (2007) stated that *B. cereus* was found to produce PHB at certain concentration of its dry cell weight, using glucose as the main carbon source. On the other hand, in accordance to research done by Vargas-García *et al*. (2007), 13 strains consist of five bacteria, one actinomycete and seven fungi had been studied for their ability to biodegrade lignocellulose. From the research it has been proven that *Bacillus licheniformis* was able to decrease the concentration of hemicelluloses, apart from showing high lignin degradation activity. Since *Bacillus licheniformis* and *Bacillus cereus* both are of the same genus, it is expected that *B. cereus* also have the ability to degrade lignin in banana stem waste almost as much as *B. licheniformis* did.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter discusses the materials and procedures for this undergraduate research project. There is four parts of experiment for this research- the revival of bacteria, fermentation analysis, delignification experiment, and delignification analysis. All of these parts are to be discussed further later in this chapter.

3.2 Revival of Bacteria

The L-dried specimens of *Bacillus cereus* was purchased from Nite Biological Resource Center (NBRC). The procedures to opening of ampules and revival of this specimen were attached together when purchased.

There were five steps in opening of ampoules and revival of L-dried specimens. First, rehydration fluid and growth medium were prepared as specified in the invoice. Second, the ampule was scored near the middle point of the cotton plug with an ampule cutter. Third, alcohol-dampened gauze was used to disinfect the ampule. Next, a sterile gauze that had not been dampened with alcohol was used to be wrapped around the ampule so as to break it carefully. Lastly, about 0.2 mL of rehydration fluid was added

to the L-dried cells by using a sterile pipette. The cell suspension was well-mixed before it was transferred to agar plate and incubated overnight at 30 ^{o}C.

3.3 Fermentation Analysis

Basically, the fermentation process was done under submerged fermentation and anaerobic conditions by using 250 mL round bottom flask bottles.

Before the delignification experiment could be run, an analysis on fermentation time of *Bacillus cereus* was done in order to figure out the lifetime of this bacterium. After regeneration, the bacteria were inoculated (10% v/v) into liquid suspension and incubated in incubator shaker for 30 hours. Next, they were inoculated again for activation purposes, and incubated in incubator shaker for another 18 hours. Finally the fermentation of *Bacillus cereus* under anaerobic conditions was done by using liquid medium without the banana stem wastes.

Samples were harvested every three hours out of 30 hours fermentation period, followed by every six hours sampling for the rest of fermentation period. Every time a sample was harvested, the absorbance value of sample was recorded. The data obtained from this analysis was used to plot the growth curve of *Bacillus cereus* in anaerobic conditions.

3.4 Fermentation for Delignification

In order to carry out the delignification experiment, the bacteria need to be activated first. The steps are the same as in fermentation analysis experiment except for banana stem waste was introduced into the medium. Figure 3.1 shows the overall workflow for the study of fermentation for delignification.

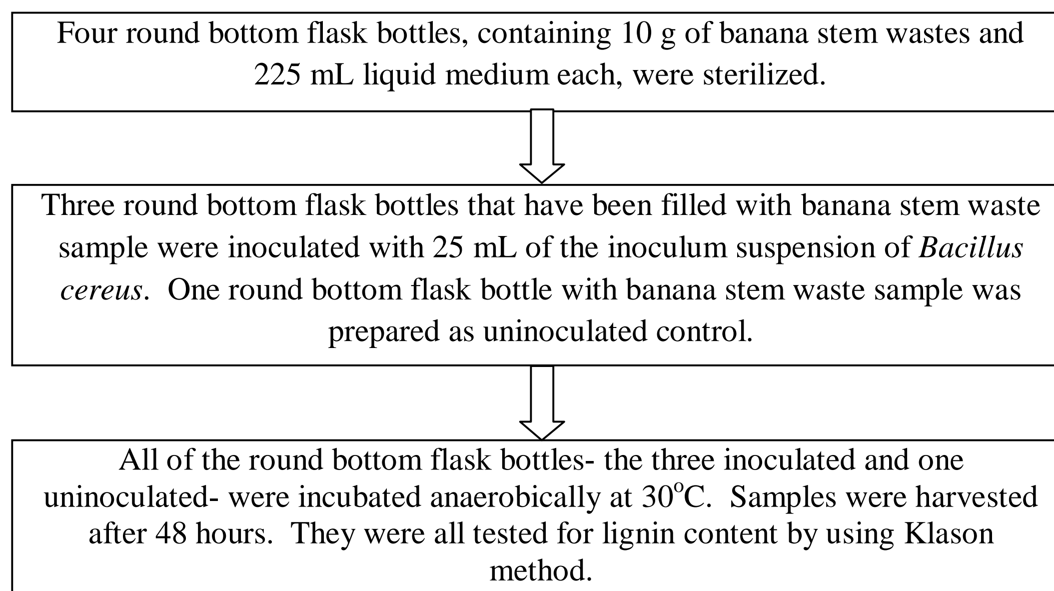

Figure 3.1: The overall workflow of study

3.4.1 Feedstock Material

The feedstock material that had been used for this undergraduate research project is banana stem waste. First, the banana stem wastes were dried under the sun. Then, they were chopped and sieved into about 20 mm pieces. The processed banana stem wastes were carefully weighed and put into four round bottom flask bottles that had been sterilized beforehand. Each flask was filled with 10 g of processed banana stem wastes. Then, 225 mL of liquid medium is added to each flask. The flasks filled with liquid medium and banana stem were sterilized before the microorganism was introduced into the flasks.

3.4.2 Rehydration Fluid and Growth Medium

The rehydration fluid, or also known as the liquid medium, was prepared in accordance to the specification for *Bacillus cereus*. The solution was prepared by heating and stirring 10 g of polypeton in 1 L of distilled water. Then, 2 g of yeast extract and 1 g of $MgSO_4.7H_2O$ were added. Once the solution was well mixed, it was kept in Schott bottles. As much as 2 L liquid medium was prepared for this study. The portion of liquid medium to be used in fermentation for delignification was directly kept in the refrigerator until it was about to be used. While the other portion of solution was sterilized in autoclave for 20 minutes at 121 ^oC.

On top of that, the growth medium, which are agar plates and agar slants were also prepared in accordance to the specification for *Bacillus cereus*. The medium was prepared by heating while stirring 10 g of polypeton in 1 L of distilled water. Then, 2 g of yeast extract, 1 g of $MgSO_4.7H_2O$ and 15 g of agar were added. When the solution was well mixed, it was poured into the Schott bottles. Next, the bottles were sterilize in autoclave for 20 minutes at 121 ^oC. After that, the agar solution was poured into Petri plates. Each of the Petri plates was covered and the agar was allowed to harden. On the

other hand, the agar solution was also poured into universal bottles and put in slanting position. The bottles were covered and the agar was allowed to harden. When all the agar in plates and bottles were harden, they were kept in refrigerator at 4 ^{o}C until they were about to be used.

3.5 Analysis for Delignification

In this undergraduate research project, the fermentation experiment was done in batch mode. This means, after setting up the experiment, it is left and only harvested at the end of the fermentation period. The aim for this experiment analysis is to estimate the percentage of lignin degraded in the banana stem wastes. Klason method, or the 72% (v/v) sulphuric acid method, was used to attain this estimation. The Klason method of analysis involves mainly three steps which are; addition of 72% (v/v) sulphuric acid, addition of distilled water, and filtration.

Before sulphuric acid could be added, the banana stem waste was filtered from fermentation flasks. It was then dried in oven at 105 ^{o}C for one hour. Next, 1 g of sample was carefully weighed and put in a 100 mL beaker. Subsequently, 20 mL of 72 % sulphuric acid was added to the beaker, and the sample was left at room temperature, about 25 ^{o}C for two hours.

In the meanwhile, a conical flask containing 500 mL of distilled water was prepared, and a water bath was set to heat to 100 ^{o}C. After the two-hour duration, the banana stem immersed in sulphuric acid was diluted in the 500 mL distilled water. It was then put in the hot water bath at shaking speed of 70 rpm for two hours.

After two hours, the samples were taken out and allowed to cool to room temperature. Then, the samples were filtrated and washed with distilled water to ensure

the samples are free from acid. Whatman glass microfiber filters were used to filter the samples. The dry weight of filters was recorded earlier. When the samples are filtered and not acidic, they were dried again in oven at 105 ^oC for one hour. The weight of samples was recorded. Samples were put in dessicator for 2 days in order to remove any moisture left.

Finally, after two days, the samples were weighed and calculations for lignin content percentage and percentage lignin degraded were done in accordance to the Equation 3.1 and Equation 3.2.

$$\text{Percentage of lignin content in sample} = \frac{\text{(Final dry weight of sample)}}{\text{(Initial dry weight of sample)}} \times 100\ \%$$

(Equation 3.1)

$$\text{Percentage of lignin degraded in sample} = \frac{\text{(\% lignin in control flask - \% lignin in sample flask)}}{\text{(\% lignin in control flask)}} \times 100\ \%$$

(Equation 3.2)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Fermentation Analysis

This chapter discusses the results obtained throughout the experiment. The first part, which is the analysis of fermentation on *Bacillus cereus* is done in order to be acquainted with the lifetime of this microorganism. Therefore, the real fermentation experiment could be done just in the period of the lifetime of *Bacillus cereus*. This is because *B. cereus* is a newly characterized, valuable organism in PHB-producing area, thus less information about it is available elsewhere.

In order to execute the fermentation analysis, the bacteria was first revived. This was done in accordance to the procedures provided together with the bacteria in ampule. The bacteria were incubated overnight on agar plate and liquid suspension at temperature 30 ^{o}C. The next day, there were colonies of bacteria observed on the agar plates, while the liquid medium suspension became cloudy which proves the existence of *B. cereus* revived.

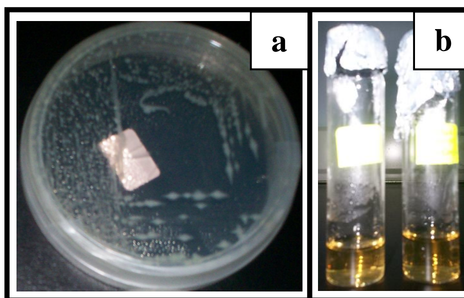

Figure 4.1: *Bacillus cereus* after being revived and incubated overnight **a**) on agar plate, and **b**) in liquid medium suspension

The next step was to culture the bacteria into liquid suspension. An inoculating loop was used to transfer three loops of bacteria's single colonies from agar plate into 100 ml liquid broth medium, shaken and incubated in incubator shaker for 30 hours at 30 ^{o}C and 250 rpm speed. This is followed by activation of bacteria, which involved the transfer of bacteria into another liquid medium with 10% v/v proportion. It is then incubated in incubator shaker for 18 hours at 30 ^{o}C and 250 rpm speed. After activation, the bacteria is said to be ready to undergo fermentation processes.

For the fermentation, the apparatus were set up to be in anaerobic conditions. 10% v/v proportion from activation flask is cultured into a round bottom flask bottle containing 225 ml of sterile medium. One round bottom flask bottle was prepared as a control. Nitrogen gas was sparged into the flasks to remove oxygen gases inside the control environment every time after harvesting. To complete the analysis, samples were harvested every three hours out of 30 hours fermentation period, followed by every six hours sampling for the rest of fermentation period. After each sampling, the samples were analyzed of the absorbance (OD) values by using UV-VIS spectrophotometer at 540 nm. Absorbance value in samples indicates the existence of *B. cereus*. Thus, sampling was stopped when the absorbance value dropped and constant. Table 4.1 shows the data collected from the fermentation analysis of *B.* cereus while Figure 4.2 depicts the graph of OD versus fermentation time which was plotted to observe the growth curve of *B. cereus*.

Table 4.1: Data for fermentation analysis of *B. cereus*.

Time		OD	
Sampling Number	**Hours**	**Blank**	***B. cereus* Anaerob**
0	2 pm	0.000	0.670
1	5 pm	0.000	0.679
2	8 pm	0.000	0.704
3	11 pm	0.000	0.796
4	2 am	0.000	0.796
5	5 am	0.000	0.865
6	8 am	0.000	0.935
7	11 am	0.000	0.872
8	2 pm	0.000	0.935
9	5 pm	0.000	0.828
10	8 pm	0.000	0.879
11	2 am	0.000	0.871
12	8 am	0.000	0.772
13	2 pm	0.000	0.646

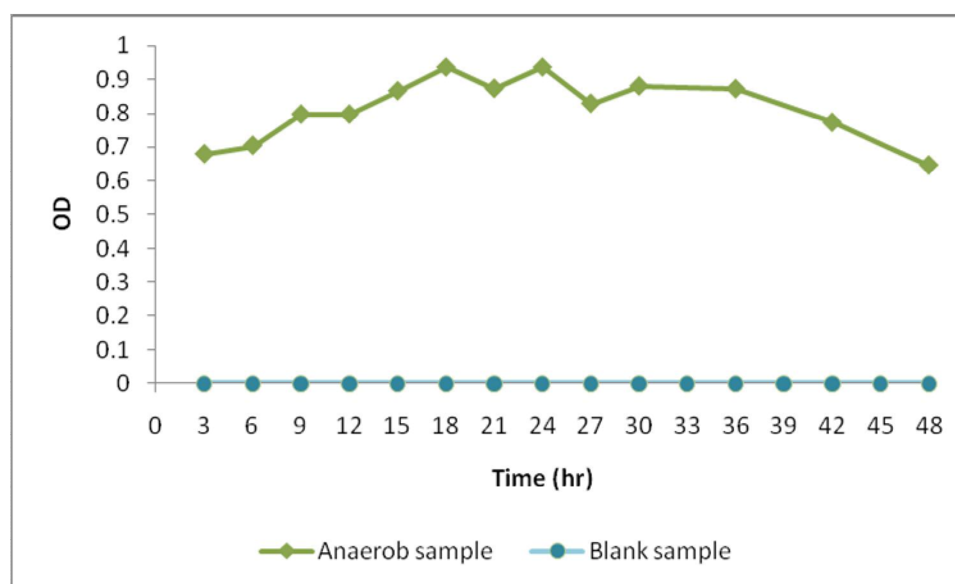

Figure 4.2: Growth curve of *Bacillus cereus* in anaerobic condition

Based on the results obtained from fermentation analysis, *Bacillus cereus* can survive up to 48 hours in anaerobic condition. Therefore, the later experiment involving the same fermentation condition should only be conducted within duration of 48 hours.

4.2 Delignification

4.2.1 Experimentation

As in the fermentation analysis experiment, the same steps applied in executing the delignification experiment, except for the preparation of medium and sample-harvesting. For delignification experiment, the processed banana stem wastes were sterilized with the fermentation medium before the bacterial broth is transferred into the flask bottles. With respect to sample-harvesting, since *B. cereus* is known to be able to survive for 48 hours, and due to the fact that the experiment was to be conducted in batch mode, once all the apparatus were readily setup and nitrogen gas was sparged, they were left in incubator at selected temperature and only harvested at the end of fermentation period. Even so, observations were made periodically to ensure that there were growth of bacteria in the flask bottles. Figure 4.3 illustrates the control's and sample's setup for anaerobic fermentation of *Bacillus cereus*.

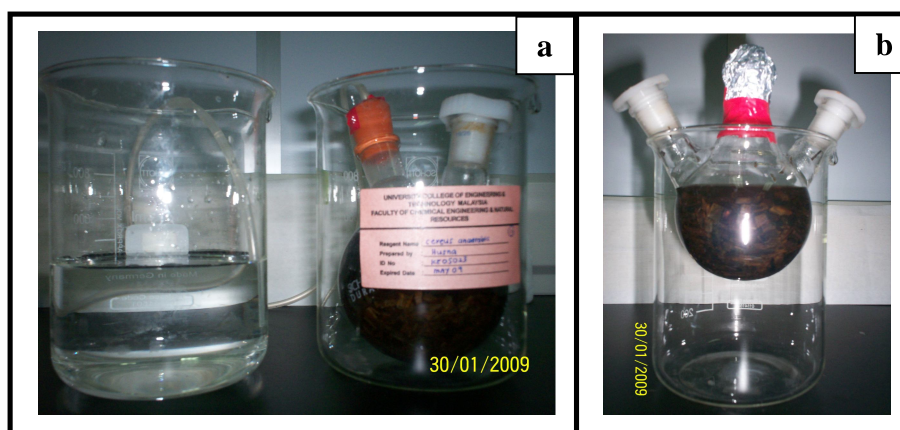

Figure 4.3: Experimental setup for anaerobic fermentation of *Bacillus cereus*; **a**) Experiment setup for sample; **b**) Experiment setup for control

For this research study, fermentation for delignification was done by comparing two temperatures- 30 ^{o}C and 37 ^{o}C. This is because *Bacillus cereus* was known to

survive in the temperature range of 30 – 37 ^{o}C. After each fermentation, analysis was done in order to determine lignin degradation activity.

4.2.2 Analysis

In this research, the method used to analyse delignification in banana stem waste was Klason method, also known as the 72% sulphuric acid method. At the end of fermentation period, all samples (banana stem waste), were harvested and filtered before they were dried in the oven at 105 ^{o}C for one hour. Then, 1 g dried banana stem from each flask bottles was carefully weighed before they were immersed in 20 ml of 72% sulphuric acid, H_2SO_4, solution and maintained at about 25 ^{o}C for two hours. The next step was to dilute the acid and put the samples into shaken waterbath at 100 ^{o}C and 70 rpm speed for another two hours. Subsequently, the samples were filtered on a pre-weighed Whatman glass microfibre filter papers. The samples were washed with distilled water in order to remove the remaining acid. Once the samples are free from acid, they were dried again in the oven at 105 ^{o}C for one hour to remove moisture. Weights of samples with filter paper before and after drying in the oven were recorded. All samples were well-labeled and put into the dessicator for two days to further remove remaining moisture. After two days, all the samples were weighed again, and the lignin content percentage, and percentage lignin degraded were calculated.

For each value of manipulated variable, which is temperature 30 ^{o}C and 37 ^{o}C, three batches of fermentation were done. Throughout the research study, the analysis was carried out two times for each batch of fermentation in equal time interval in order to get hold of accurate and convincing results.

4.2.2.1 Analysis for Fermentation at 30 ^{o}C

Table 4.2 and 4.3 shows the analysis results of delignification of banana stem by *Bacillus cereus* in anaerobic condition at 30 ^{o}C. The calculations for lignin content were done by using Equation 3.1 while the calculations for lignin degradation were done by using Equation 3.2.

Table 4.2: Percentage lignin degraded at 30 ^{o}C (Analysis 1_{30})

Sample Name	Dry Weight, g		Lignin content, %	Lignin Degradation, %
	Initial	Final		
Control 1	1.0072	0.0566	5.62	24.02
Sample 1	1.0031	0.0428	4.27	
Control 2	1.0013	0.1283	12.8	-31.64
Sample 2	1.0005	0.1686	16.85	
Control 3	1.0072	0.0566	5.62	-68.33
Sample 3	1.0035	0.0949	9.46	

Data at Analysis 1_{30} as in Table 4.2 state that from the three batches of fermentation carried on, one of the results brings positive and reliable value while the other two results came out negatively due to errors done during handling the experiment. Analysis from fermentation batch 1 suggests that lignin degraded was 24.02 %. Analysis from fermentation batches 2 and 3 suggest percentage of lignin degradation of -31.64 % and -68.33 % respectively. The reason for the negative percentage is the way the experiment was conducted. For instance, the banana stem was not completely dried when the analysis was carried on. Other than that, the acid might not completely washed out during the washing step in the analysis. Nonetheless as a conclusion for this analysis, the percentage of lignin degradation is 24.02 %.

Table 4.3: Percentage lignin degraded at 30 ^{o}C (Analysis 2_{30})

Sample Name	Dry Weight, g		Lignin content, %	Lignin Degradation, %
	Initial	Final		
Control 1	1.0053	0.2706	26.92	2.67
Sample 1	1.0049	0.2633	26.20	
Control 2	1.0053	0.2706	26.92	3.53
Sample 2	1.0175	0.2642	25.97	
Control 3	1.0053	0.2706	26.92	1.71
Sample 3	1.0025	0.2653	26.46	

For the same three batches of fermentations, another analysis was carried on in order to be more accurate in determining the results. The set of data collected is named as Analysis 2_{30} as shown in Table 4.3. There was improvement on the way the analysis was handled so as to minimize and possibly avoid any error during experiment. Based on the data collected, analysis on fermentation batches 1, 2 and 3 resulted in 2.67, 3.53 and 1.71 % of lignin degraded, respectively. As a conclusion for Analysis 2_{30}, the average percentage of lignin degraded is 2.64 %.

Based on the data and results gained, in anaerobic condition, *Bacillus cereus* was able to degrade lignin about 13.3283 % in banana stem waste at 30 ^{o}C.

4.2.2.2 Analysis for Fermentation at 37 °C

Table 4.4 and 4.5 shows the analysis result for delignification of banana stem by *Bacillus cereus* in anaerobic conditions at 37 °C. As in previous section, the calculations for lignin content were done by using Equation 3.1 while the calculations for lignin degradation were done by using Equation 3.2.

Table 4.4: Percentage lignin degraded at 37 °C (Analysis 1_{37})

Sample Name	Dry Weight, g		Lignin content, %	Lignin Degradation, %
	Initial	Final		
Control 1	1.0053	0.2063	20.52	1.56
Sample 1	1.0004	0.2021	20.20	
Control 2	1.0288	0.2404	23.37	4.99
Sample 2	1.0018	0.2224	22.20	
Control 3	1.0067	0.2159	21.45	-2.24
Sample 3	1.0049	0.2204	21.93	

Table 4.4 shows the first analysis result for fermentation at 37 °C. From the table, the percentage of lignin degradation is 1.56 and 4.99 % for fermentation batches 1 and 2. Meanwhile for fermentation batch 3, the result is -2.24 %. This is caused by error done during the handling of experiment. The possible errors include the incompleteness washing of sulphuric acid during the washing step. If the acid was not completely washed, there was possibility for polysaccharide compound to still be available thus disturbing the actual dry weight of lignin. Even so, the average percentage of lignin degraded at 37 °C for Analysis 1_{37} is calculated to be 3.275 %.

Table 4.5: Percentage lignin degraded at 37 ^{o}C(Analysis 2_{37})

Sample Name	Dry Weight, g		Lignin content, %	Lignin Degradation, %
	Initial	Final		
Control 1	1.0003	0.1862	18.61	-33.85
Sample 1	1.0012	0.1926	19.24	
Control 2	1.0022	0.1895	18.91	-78.79
Sample 2	1.0082	0.2057	20.40	
Control 3	1.0005	0.3044	30.42	2.96
Sample 3	1.0008	0.2954	29.52	

For second analysis at 37 ^{o}C, the results show lignin degradation percentage as much as -33.85 and -78.79 % for fermentation batches 1 and 2, while 2.96 % for fermentation batch 3. The high percentage of negative values indicate mishandlings during experiment, among of which could be the use of filter paper selected. The wrong choice could end up in tearing up or burning of filter paper which will eventually effect the dry weight of lignin content thus affecting lignin degradation percentage. Another error can be the incomplete drying of banana stem, apart from neutralization of banana stem by washing with distilled water. As conclusion for Analysis 2_{37}, the average percentage of lignin degraded is 2.96 %.

Based on the data and results gained, in anaerobic condition, *Bacillus cereus* was able to degrade lignin about 3.1175 % in banana stem waste at 37 ^{o}C.

After carry out the analysis at 30 and 37 ^{o}C, it can be concluded that delignification of banana stem waste in anaerobic condition was done better at 30 ^{o}C with average percentage of 13.3283 % lignin degraded compared to that of 37 ^{o}C with average percentage of 3.1175 % lignin degraded. This is in fact a good result because it means that in anaerobic conditions, less temperature thus less energy is needed for *Bacillus cereus* to delignify banana stem waste.

4.2.3 Error and Precautions

In the efforts of performing the analysis of delignification, there were several errors that could be highlighted and concerned of for future study. Firstly, the banana stem waste used should be from the same source and type of banana. This is to make sure the content of lignin to be delignified are about the same amount thus a more precise comparison could be done.

Secondly, the type of filter paper used to filter the sample should be given attention. The filter should neither be easily tear-up when being washed repeatedly with water, nor burnt when put in the oven at a temperature of 105 ^{o}C. This is crucial because the weight of filters were recorded before being used so as to obtain the final weight of sample later on. Any changes on filters such as tear-up or burnt could affect their dry weights, thus affecting the weight of sample and eventually, affecting the lignin content. Whatman glass microfiber filters 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 105 ^{o}C after being used to filter the samples.

Finally, in the washing step where the samples were rinsed with distilled water repeatedly, it is important to ensure all the acids are washed before the samples were dried in the oven. In other words, the samples need to be neutral in pH before proceeding to further steps. This is because, sulphuric acid dissolves all polysaccharides compound in the banana stem sample, and if there was still acid left in the sample, it suggested that the sample contain lignin and polysaccharides, instead of lignin alone. Once again, this could lead to inaccuracy of lignin content analysis since the weight of the sample was obviously affected.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

To wrap up this undergraduate research thesis, submerged fermentation in anaerobic conditions of banana stem waste have been done using *Bacillus cereus*. Results showed that *Bacillus cereus* was able to survive for as long as 48 hours in anaerobic conditions. In addition to that, analysis of lignin degradation, or simply delignification, proved that *Bacillus cereus* has low ability to delignify banana stem waste in anaerobic conditions. Data collected verifies that lignin degradation activity was optimum at 30 ^{o}C with average lignin degradation percentage of 13.33 %. This value is far from the results of fermentation for *Bacillus cereus* in aerobic conditions which suggest that the optimum temperature for delignification was 37 ^{o}C with average lignin degradation percentage of 28.69 %. This study proves that *Bacillus cereus* can delignify lignin better at aerobic conditions.

5.2 Recommendation

Based on the results and conclusion of this study, some recommendations for future work comprises of certain aspects. In order to make this study more interesting, the parameters of study such as pH and agitation speed should be introduced so that the results can be clearly justified and proved. In addition, for each batch of fermentation, the raw materials used should be from the same source. Variety of sources for one batch of fermentation may lead to inaccuracy since the content of lignin is different for each type of banana stem used.

Conversely, a method such as specific agar method should be employed so as to prove the existence of *Bacillus cereus* previously cultured on agar plates. On top of that, one should be precise in conducting experiments in terms of interval time. This is mainly because most of the parts in this study, especially the analysis part, involve real adjusting timing. Abiding the time interval will in turn give a more accurate and convincing results.

Finally, it is also suggested that, in executing the Klason method analysis, Whatman fiber glass filter paper should be used instead of Whatman ashless or qualitative filter papers. This is because fiber glass filter paper will not be burnt when it is put in high temperature in oven, thus it will not disturb the dry weight of filter paper or the sample. Hence this will ease the calculation to obtain lignin degradation activity.

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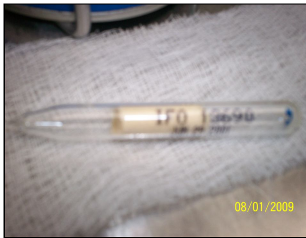
APPENDIX A

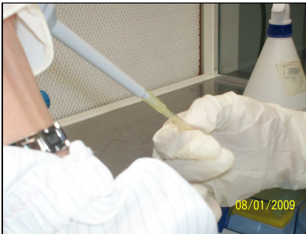
REVIVAL OF *Bacillus cereus*



L-dried cells of *Bacillus cereus* in the ampule.

Step 1: Scoring the ampule near the middle-point of the cotton plug with an ampule cutter.

Step 2: Wrapping sterile gauze around the ampule and carefully break it.



Step 3: Addition of rehydration fluid to the L-dried cells with a sterile pipette.

Step 4: Mix well the cell suspension.

Step 5: Transfer of cell suspension to a growth medium (i.e. agar plate).

APPENDIX B

CALCULATION FOR PREPARING 72% SULPHURIC ACID SOLUTION

- Minimum final volume of solution needed = 20 mL * 4
  = 80 mL
- Final concentration needed, M_2 = 0.72 M of H_2SO_4
- Final volume of solution needed, V_2 = 100 mL
- Initial concentration available, M_1 = 0.98 M of H_2SO_4

By using the dilution formula;

$$M_1V_1 = M_2V_2$$

$$V_1 = (0.72 \text{ M})(100 \text{ mL}) / (0.98 \text{ M})$$

$$= 73.4694 \text{ mL}$$

$$\approx 73.5 \text{ mL } H_2SO_4 \text{ 98\% v/v}$$

Thus, the amount of distilled water needed;

= (100 – 73.5) mL

= 26.5 mL distilled water

APPENDIX C

KLASON METHOD OF ANALYSIS

Samples of banana stem waste that has been filtered after fermentation.

Samples in shaking waterbath.

Filtration of samples by manual filtration.

Checking the acidity of sample by using Litmus paper.

APPENDIX D

THE EQUIPMENTS USED IN RESEARCH STUDY

Appendix D.1.: Laminar air Flow Cabinet (Model AHC-4A1)

Appendix D.2: Double Stack Shaking Incubator Infors

Appendix D.3: Shaking Water Bath (Model BS-21)

Appendix D.4: UV-Visible Single Beam Spectrophotometer (Model U-1800)