

**PRODUCTION OF POLY-HYDROXY BUTYRATE (PHB) BY *Bacillus*
Cereus USING BANANA STEM AS A SUBSTRATE**

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ABSTRACT

The research was focused on delignification process and production of (Poly- β -hydroxybutyrate) PHB by *Bacillus cereus* using banana stem wastes as substrate. This study is divided into three parts which are determination growth curve of *Bacillus cereus*, lignin analysis using Klason method and determination of PHB. Delignification process is functioning to degrade lignin from banana stem to get cellulose that can be used as carbon sources in production of PHB. PHB is a carbon storage polymer that is widely distributed among prokaryotes including *Bacillus cereus*. The delignification methods are divided into three parts which are preparation of raw material and microorganisms, fermentation process and lignin analysis. Fermentation was carried out aerobically at optimum temperature 37 °C and 250 rpm of agitation speed for 2 days. In this process, Klason method was used to identify the percentage of lignin degraded. Based on the analysis, it shows that *Bacillus cereus* is able to degrade lignin from banana stem wastes. The average lignins degrade by *Bacillus cereus* was 40.175%. Meanwhile, for production of PHB methods were divided into four parts which are fermentation, pre-treatment, extraction and analysis. In extraction process, ultrasonic treatment, H₂SO₄, HCl and chloroform was used to break the granule and cell wall. In this study, the best PHB production and percentage yield of this strain was determined. The PHB production was 27.897 mg/ml and the percentage yield was 55.48% at 48 hours. After 48 hours there was a decrease in PHB yield because of the bacteria used PHB as a source of carbon and nitrogen, causing an unsuitable condition due to inadequate nitrogen and carbon sources in the medium.

ABSTRAK

Proses pengurangan lignin dan penghasilan PHB oleh *Bacillus cereus* dengan menggunakan batang pisang telah di kaji. Kajian ini terbahagi kepada tiga bahagian iaitu, penentuan graf pertumbuhan bagi *Bacillus cereus*, analisis lignin menggunakan kaedah Klason dan penghasilan PHB. Proses pengurangan lignin bertujuan untuk mengurangkan lignin dalam batang pisang supaya kandungan selulosa di dalamnya boleh digunakan sebagai sumber karbon dalam penghasilan PHB. PHB adalah salah satu polimer penyimpan karbon dimana digunakan oleh semua prokaryot termasuk *Bacillus cereus*. Proses pengurangan lignin terbahagi kepada tiga bahagian penyediaan bahan mentah dan mikroorganisma, proses fermentasi, dan analisis lignin. Proses fermentasi dijalankan secara aerobik pada suhu optimum 37 °C dan dengan kelajuan putaran 250 rpm selama dua hari. Kaedah Klason digunakan untuk mengenalpasti peratusan pengurangan lignin. Hasil analisis menunjukkan *Bacillus cereus* berkeupayaan untuk mengurangkan lignin dalam batang pisang. Purata kadar pengurangan lignin oleh *Bacillus cereus* dalam kajian ini adalah sebanyak 40.175%. manakala untuk penghasilan PHB pula terbahagi kepada empat bahagian iaitu rawatan ultrasonik, H₂SO₄, HCl dan klorofom digunakan untuk memecahkan granul dan dinding sel. Dalam kajian ini, peratusan penghasilan PHB yang paling tinggi oleh bacteria ini telah dikenal pasti. Penghasilan tertinggi ialah 27mg/ml dan peratus penghasilan ialah 55.48% pada jam ke 48. Selepas jam ke 48, penghasilan PHB menurun kerana pada waktu itu bacteria menggunakan PHB sebagai sumber karbon dan nitrogen, yang menyebabkan keadaan yang tidak sesuai kesan daripada kekurangan sumber nitrogen dan karbon di dalam media.

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CHAPTER 1

INTRODUCTION

1.1 Background of the Study

In Malaysia, banana had been produced in a large quantity which is the total plantation area is 33 704.2 hectares (Abdul Khalil *et al.*, 2006). Banana consists of stem that call as pseudostem. After harvesting, those pseudostems are lefted waiting to be degraded naturally. According to Abdul Khalil *et al.*, (2006), the content of cellulose is high (> 40%) in pseudostem. However, those pseudostems have high content of lignin. Lignin provides plant tissue and individual fiber with compressive strength and stiffens the cell wall of the fibers, to protect the carbohydrates from chemical and physical damage (Saheb and Jog, 1999). Lignin is an undesirable polymer, and its removal during pulping requires high amount of energy and chemicals (Abdul Khalil *et al.*, 2006). Therefore, delignification had taken place to delignify the pseudostem. The delignification process is carried out using microorganisms. Instead of waste disposal problem, the rapid growth of plastic consumption was also accounted. Improper disposal of plastics has threatened natural environment worldwide since long time ago. Conventional petrochemical plastics are recalcitrant to microbial degradation (Flechter, 1993). Excessive molecular size seems to be mainly responsible for the resistance of these chemicals to biodegradation and their persistence in soil for a long time (Atlas, 1993). These non-degradable to petrochemical plastics accumulate in environment at a rate of 25 million tones per year (Lee *et al.*, 1991). To overcome this problem, the

production and applications of eco-friendly products such as bio-plastics becomes inevitable. Typically, the bio-plastic production can be made by specific bacteria such as *Alcaligenes latus*, *Azotobacter vinelandii*, *methylophs*, *pseudomonas olevorans* and etc. Polyhydroxyalkanoates (PHAs) which produced mainly by bacteria provide a degradable alternative to petrochemical plastics (Anderson and Dawes, 1990; Kumar *et al.*, 2004).

PHA have been attracting considerable attention as biodegradable plastics due to their similar properties to various thermoplastics and elastomers, which have been used in consumer products, and completely degraded to water and carbon dioxide upon disposal under various environments (Lee and Choi, 1999). However, the use of PHA in a wide range of applications has been hampered by their high production cost. This is mainly due to the expensive carbon substrates and tedious production procedures (pure cultures) (Yamane *et al.*, 1996; Lee and Choi, 1998; Grothe *et al.*, 1999). In order to reduce their production cost, mixed cultures and cheap carbon sources have been used. Several inexpensive carbon substrates such as molasses, whey, cellulose, plant oils and hydrolysates of starch (corn and tapioca) can be excellent substrates for the bacteria utilizing them to produce PHA (Lee and Choi, 1999). Waste-based substrates have only recently been recognized for PHA production (e.g. Pozo *et al.*, 2002; Wong *et al.*, 2002; Carucci *et al.*, 2001) because plastic producers are currently working towards decreasing the cost of bio-polymers. Polyhydroxybutyrate (PHB) is one of the members of PHA that is widely produced.

Novel technologies have been discovered to produce PHAs from organic matters in wastewater (Chua *et al.*, 1997; Mohad *et al.*, 1996; Punrattanasin, 2001), industrial wastes (Rusendi *et al.*, 1995; Wong and Lee, 1998), municipal and food wastes (Du and Yu, 2002; Takabatake *et al.*, 2002; Lee and Gilmore, 2005). Hence, replacement of non-biodegradable with biodegradable plastic from organic waste can provide multiple benefits to the environment and contribute to sustainable development (Du and Yu, 2002).

1.2 Significant of Study

As an alternative for managing disposal waste from banana plantation, we are going to use a banana stem waste as a carbon source by delignification process for production of PHB. Therefore, the strategies to upgrade and to optimize the PHB production using a renewable carbon sources from banana stem waste combined with mixed cultures has been chosen as research goal. Meanwhile, the pollution loads will be reduced significantly.

There are several important items to be considered. Those will benefit to achieve the objectives of this study, which are:

- i. Plastics have been an integral part of our life. However, disposal of these non-biodegradable (petrochemical derived) plastics poses a threat to our environment. The current worldwide demand for plastics is in excess of 100 million tones per year (Abou Zeid, 2001). Therefore, replacement of non-biodegradable by degradable plastics will help to prevent environmental problems.
- ii. The main purpose is to produce bioplastic that is environmental friendly. By using the organic plant wastes like banana stem (as raw materials) to produce cellulose (as carbon source) it is indirectly prevented another caused of pollution. Besides that, it is to prove that all the component of banana plant itself is useful including the stem. Other than that, wastes can play the role as carbon source instead of using food materials. The cost for the production of bioplastic will be decrease when using wastes as the source of the carbon source for PHB production.

1.3 Objective

To determine the potential of *Bacillus Cereus* for PHB production by using banana stem waste as a substrate.

1.4 Scope of Study

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

- i. Delignification of banana stem waste by *Bacillus Cereus* at optimum temperature 37°C, rotation 250rpm, ph7 and time (48hrs). (Nazime Mercan (2002))
- ii. Production of PHB by *Bacillus cereus* using banana stem waste as a carbon sources. 37°C, rotation 250rpm, ph7 and time (48hrs). (Nazime Mercan (2002))

1.5 Problem Statement

Our whole world seems to be wrapped in plastic. Almost every product we buy most of the food we eat and many of the liquids we drink come encased in plastic. In Australia around 1 million tonnes of plastic materials are produced each year and a further 587,000 tonnes are imported. Packaging is the largest market for plastics, accounting for over a third of the consumption of raw plastic materials – Australians use 6 billion plastic bags every year. Plastic packaging provides excellent protection for the product, it is cheap to manufacture and seems to last forever. Lasting forever, however, is proving to be a major environmental problem. Another problem is that traditional plastics are manufactured from non-renewable resources – oil, coal and natural gas.

In an effort to overcome these shortcomings, biochemical researchers and engineers have long been seeking to develop biodegradable plastics that are made from renewable resources, such as plants.

Another way of making biodegradable polymers involves getting bacteria to produce granules of a plastic called polyhydroxyalkanoate (PHA) inside their cells. Bacteria are simply grown in culture, and the plastic is then harvested. Going one step further, scientists have taken genes from this kind of bacteria and stitched them into corn plants, which then manufacture the plastic in their own cells.

Unfortunately, as with PLA, PHA is significantly more expensive to produce and, as yet, it is not having any success in replacing the widespread use of traditional petrochemical plastics. Indeed, biodegradable plastic products currently on the market are from 2 to 10 times more expensive than traditional plastics. But environmentalists argue that the cheaper price of traditional plastics does not reflect their true cost when their full impact is considered. For example, when we buy a plastic bag we do not pay for its collection and waste disposal after we use it. If we added up these sorts of associated costs, traditional plastics would cost more and biodegradable plastics might be more competitive.

So the aimed of this project is to produce a cheaper biodegradable plastic using banana waste as a carbon sources. Where it also can help in managing waste disposal in our country.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter reviews on the PHB production using *Bacillus Cereus* and banana stem waste as raw material to produce carbon source. The scopes covers capability of *Bacillus Cereus* in producing PHB using banana stem as substrate, fermentation process, development of plastic materials, polyhydroxybutyrates (PHBs) and lignin degradation of Banana Stem Waste. In this chapter, these topics are reviewed to provide the basic insight to the rudiments of the studies of PHB production using *Bacillus Cereus*.

2.2 Banana Stem Waste

In Malaysia, banana had been produced in a large quantity which is the total plantation area is 33 704.2 hectares (Abdul Khalil *et al.*, 2006). Banana consists of stem that call as pseudostem. After harvesting, those pseudostems are lefted waiting to be degraded naturally. According to Abdul Khalil *et al.*, (2006), the content of cellulose is high (> 40%) in pseudostem. However, those pseudostems have high content of lignin. Lignin provides plant tissue and individual fiber with compressive strength and stiffens the cell wall of the fibers, to protect the carbohydrates from chemical and physical damage (Saheb and Jog, 1999). Lignin is an undesirable polymer, and its removal

during pulping requires high amount of energy and chemicals (Abdul Khalil *et al.*, 2006). Therefore, delignification had taken place to delignify the pseudostem. The delignification process is carried out using microorganisms. Therefore it is important to determine the effectiveness of microorganisms such as *Bacillus cereus* in delignification process.

2.3 Development of Plastic Materials

Plastics have been an integral part of our lives. From automobiles to medicine, plastics are utilized in almost every manufacturing industry in the world. Approximately 25 million tons of plastics are produced by the plastics industry every year (Wong *et al.*, 2002). Plastics are advantageous because as synthetic polymer, their structure can be chemically manipulated to have a wide range of strengths and shapes. Plastics can be easily molded into any desired shape including fibers and thin films. They also have a high chemical resistance and are quite elastic. Therefore, plastics are very popular in many durable, disposal goods and as packaging materials. Plastics are truly significant in our industrial society.

The development of biodegradable plastics has become one of the major concerns in the present society because the disposal of the plastics has pointed out their major weaknesses. Plastics are produced from non-renewable resources such as petrochemical and are not compatible with natural carbon cycles because of their non-degradable characteristics. Plastics have molecular weights ranging from 50,000 to 1,000,000 Dalton. These excessive molecular weights, which provide durability seems to be mainly responsible for the resistance of these chemicals to biodegradation. They can persistence in soil for a long time. Due to the non-biodegradability of petrochemical-derived plastics, several ways of managing the used-plastics have been initiated. Incineration has been one of the options in dealing with non-degradable plastics. But the process can be expensive and dangerous because harmful chemicals like hydrochloric acid and hydrogen cyanide are released during incineration. Another

alternative is recycling, but it also presents some major disadvantages, as it is difficult to sort the wide variety of plastics and there are also changes in the plastic's material such that its further application range is limited. The safest and least expensive ways to deal with the disposal of non-degradable plastics is landfill disposal. However, landfills are rapidly reaching their maximum capacity while the amount of plastic thrown away is rising. Besides, plastics can cause ecological problem. For example, several hundred thousand tons of plastics are discarded into marine environment every year and accumulate in certain oceanic regions. The marine animals may kill every year, either by choking on plastics they mistake as food or becoming entangled in non-degradable plastics debris. This may result in extinction of certain marine animals and damage the beautiful natural scenery.

In recent years, there has been increasing public concern about the harmful effect of the disposal of petrochemical-derived plastic materials in the environment. When discarded into nature, conventional plastics can persist for many decades, at best a mere eyesore, at worst posing threat to wildlife. Experts also anticipated the future non-availability of the non-renewable petroleum raw material for existing plastic products. Therefore, replacement of non-biodegradable by biodegradable plastic is of major interest both to decision-makers and the plastic industry. Making eco-friendly products such as bio-plastics is one such reality that can help us to overcome the problem. Biodegradable plastics which also known as green plastics are plastics made from biodegradable polymer materials. Polyhydroxybutyrate (PHB) is a suitable source for biodegradable polymer material because their fully degradability and non-pollutant characteristics. Braunegg *et al.* (1998) defined biodegradability as the capability to be broken down, especially into innocuous products by the action of living things, known as microorganism. Bacteria and fungi are the natural occurring microorganisms that are capable to degrade PHA to carbon dioxide and water through secreting enzymes. It can also be degraded through non-enzymatic hydrolysis.

2.4 Polyhydroxybutyrates (PHBs)

Polyhydroxybutyrate (PHB), a lipid-like polymer of 3-hydroxybutyrate, is a representative member of polyhydroxyalkanoates (PHAs) formed in many bacteria. They accumulate as a carbon and energy reserves under unbalanced (unfavorable) growth conditions, such as nutrient limitation (Wang and Yu, 2000). PHB is the most common and best-characterized PHA stored by bacteria. Other frequently stored PHAs are polyhydroxyvalerate or polyhydroxyhexanoate. Their presence and relative proportions depend on the type of carbon substrate used by the microorganism. Bacteria that have been shown to efficiently produce PHB include *Alcaligenes eutrophus*, *Alcaligenes latus*, *Azotobacter vinelandii*, and recombinant *Escherichia coli*.

PHB is a partially crystalline polymer which has material properties similar to polypropylene (PP) and polyethylene (PE) (Howells *et al.*, 1982; Holmes *et al.*, 1988; Lee, 1996). Therefore, PHB has been considered as one of the most promising biodegradable plastics and as an alternative to petrochemical plastics. This is due to their biocompatibility, biodegradability and versatile properties make it an eco-friendly substitute for synthetic polymers (Mergaert *et al.*, 1992; Brandl *et al.*, 1995). In addition, PHB has more advantages because it is far less permeable than PE and PP, this known as a better material for food packaging needless to use antioxidant.

However, industrial application of PHB has been hampered owing to its low thermal stability and excessive brittleness upon storage (Matsusaki *et al.*, 2000). Due to the poor physical properties of PHB, the incorporation of a second monomer unit into PHB can significantly enhance its properties. This has led to an increased interest to produce hetero-polymers with improved qualities. The incorporation of 3-hydroxyvalerate (3HV) into the PHB has results in a poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] which is more flexible and tougher than PHB, and more easier to degrade when discarded into the natural environment.

On the other hand, the use of PHB in a wide range of applications has been hampered mainly by their high production cost compared with conventional petrochemical-based polymers (Choi and Lee, 1999). For the commercialization of PHAs, a great deal of effort has been devoted to reduce the production cost by the development of better bacterial strains and more efficient fermentation/recovery processes. There are several factors that can affect the production cost of PHB such as PHB productivity, content and yield, the cost of the carbon substrate and the recovery method. From literature, the major cost in the PHB production is the cost of the substrate. Therefore, the use of cheaper carbon sources can lower the production cost of PHB. A research on the cost of various carbon sources for PHB production is given in Table 2.2 (Lee *et al.*, 1996b; Madison and Huisman, 1999). It can be concluded that yields of PHB from various substrates are similar (except the last one that might have a low PHB production). The price of substrate has the largest influence on the raw material cost for production of PHB. It can be seen that the cheapest substrate cost is 0.22 USD/kg PHA (biodegradable plastic) compared with 0.185 USD/kg polypropylene (petrochemical plastic) (Kothuis and Schelleman, 1998). Thus, cafeteria wastes as carbon source is an alternative to reduce the production cost of PHB because it is waste-based and rich in organic carbon.

Table 2.1: Effect of substrate cost and PHB yield on the production cost of PHB

Substrate	Price (USD kg ⁻¹)	Yield (g PHB /g Substrate)	Substrate Cost (USD kg ⁻¹ PHB)
Glucose	0.493	0.38	1.350
Sucrose	0.295	0.40	0.720
Methanol	0.180	0.430	0.420
Acetic acid	0.595	0.380	1.560
Ethanol	0.502	0.500	1.000
Cane molasses	0.220	0.420	0.520
Cheese whey	0.071	0.330	0.220
Hydrolyzed corn starch	0.220	0.185	0.580

Hemicellulose hydrolysate	0.069	0.200	0.340
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2.5 Microorganism

Bacillus cereus is a spore-forming organism which occurs naturally in most foods. It causes two different forms of food poisoning: an emetic illness and a diarrhoeal illness. The emetic illness is mediated by a highly stable toxin that survives high temperatures and exposure to trypsin, pepsin and pH extremes. The diarrhoeal illness is mediated by a heat- and acid-labile enterotoxin.

2.5.1 Growth:

- Temperature: Optimum 30-37°C. Some strains can grow up to 55°C while others can grow as low as 4-5°C. Many strains from dairy products are able to grow at low temperatures.
- pH: The minimum pH for growth is 4.3, maximum pH around 9.3.
- Growth is inhibited in the presence of 0.1% acetic acid (pH 5.1).
- Atmosphere: Growth is best in the presence of oxygen. Grows well anaerobically. Toxin production is lower under anaerobic conditions.
- Water activity: Minimum range of water activity for vegetative growth is 0.912-0.950.

2.5.2 Survival:

- Temperature: *Vegetative cells* are readily killed by heat but spores are moderately heat resistant. Heat resistance is increased in high-fat and oily foods

(insoybean oil, the D time at 121°C is 30 min). Higher heat resistances also occur in foods with low water activity. *Spores* are more resistant to dry heat than moist heat. *Emetic toxins* are extremely resistant to heat (can survive 90 min at 126°C). Diarrhoeal toxins are inactivated at 56°C in 5 min.

- pH: *B. cereus* organisms die suddenly in yoghurt when the pH reaches 4.5. Emetic toxin survives extremes of pH (2-11)
- Water Activity: Spores survive for long periods in dried foods.

2.5.3 Inactivation (CCPs and Hurdles):

- Temperature: *For spores*: D₈₅ = 33.8-106 min. D₉₅ ranged from 1.5-36.2 min in distilled water and 1.8-19.1 min in milk. Considerable variation was observed between different strains.
- pH: Inactivated by 0.1 M acetic, formic and lactic acids in nutrient broth.
- Water activity: 7.5% NaCl inhibits growth.
- Preservatives: (NB: Some of the preservatives discussed here may not be permitted in New Zealand). Growth is inhibited by 0.26% sorbic acid at pH 5.5 and 0.39% potassium sorbate at pH 6.6. The addition of 0.2% calcium propionate prevents germination of *B. cereus* in bread. Nisin is commonly used to inhibit germination and spore growth in processed cheese, dairy desserts, canned foods, cured meats and high moisture baked products such as crumpets and pikelets. A level of 3.75 µg/g of nisin in crumpet batter was effective. Other antimicrobials which have an effect on *B. cereus* include benzoate, sorbate, ethylenediaminetetraacetic acid (EDTA) and polyphosphates. Preserving foods in modified atmospheres has been shown to control the growth of *B. cereus*. Preservatives can be applied at reduced levels to inhibit the growth of *B. cereus* when used in combination (hurdle effect). Sanitisers/Disinfectants: Most chemical sanitizer used routinely in the food industry will destroy *B. cereus* on surfaces.

- Radiation: Spores are more resistant to radiation than vegetative cells. Spores are more sensitive to heat after preirradiation at 4kGy before heating at 90oC.

2.5.4 The Illness

B. cereus-associated foodborne illness occurs as 2 distinct syndromes: emetic and diarrhoeal.

- Incubation: *Emetic*; 1-6 hours after eating contaminated food. *Diarrhoeal*; 10-12 hours.
- Symptoms: The symptoms of the emetic syndrome result from ingestion of pre-formed toxin: nausea and vomiting, occasionally followed by diarrhea (similar to *S. aureus*). diarrhoeal symptoms results from ingestion of vegetative organisms or spores and their subsequent multiplication and toxin production within the intestinal tract: abdominal pain, watery diarrhoea and occasional nausea (similar to *C. perfringens*). recovery is rapid for both syndromes, usually within 12-24 hours. Very few fatalities have been reported.
- Condition: Gastroenteritis.
- At Risk Groups: All people are believed to be susceptible to intoxication and infection but the intensity of symptoms may vary between individuals.
- Long Term Effects: None
- Dose: Large numbers, $>10^5$ /g of food, are required to produce toxin or cause infection. No food containing $>10^4$ *B. cereus* /g can be considered safe for consumption. Small numbers of *B. cereus* in food are not a direct hazard to health
- NZ Incidence: In the Annual Summary of Outbreaks in NZ (1999) 16 outbreaks were attributed to *B. cereus* (45 cases), 6 outbreaks in 1998 (21cases).
- Treatment: Usually no treatment is given. Fluids may be administered when diarrhoea and vomiting are severe.

2.5.5 Sources

- Human: Humans are not a significant source of food contamination by *B. cereus*. This organism already exists on many foods and can therefore be transiently carried in the intestine of healthy humans (0-43%).
- Animal: Animals can carry *B. cereus* on parts of their body. May occasionally cause mastitis in cows.
- Food: Raw foods of plant origin are the major source of *B. cereus*. The widespread distribution of the organism, the ability of spores to survive dried storage and the thermal resistance of spores, means that most ready-to-eat foods will contain *B. cereus* and will require control measures to prevent growth, especially after cooking has eliminated competing flora. Strains producing emetic toxin grow well in rice dishes and other starchy foods, whereas strains producing diarrhoeal toxin grow in a wide variety of foods from vegetables and salads to meat and casseroles. Numerous dried herbs and spices and dehydrated foods have been shown to contain *B. cereus*.
- Environment: *B. cereus* is widely distributed in nature and can be found in soil, dust, air, water and decaying matter. Its ability to form spores allows survival through all stages of food-processing, other than retorting.
- Transmission Routes: Ingestion of contaminated food.

2.5.6 Outbreaks and Incidents

- Outbreaks: Most *B. cereus* food poisoning incidents result from eating cooked foods which are cooled slowly and stored incorrectly. Outbreaks of emetic-type illness generally result from eating rice products or starchy foods such as potato and pasta. Outbreaks of diarrhoeal-type illness result from eating foods in which *B. cereus* organisms have grown to large numbers.

- *New Zealand* examples:

Fried rice, meat stew, vegetables: 30 cases. Control measure failure: possibility of inadequate cooling, prolonged storage at incorrect temperatures.

Chinese takeaway meal: 7 cases. Control measure failure: inadequate cooling and storage of rice.

Pancakes: 5 cases. Control measure failure: prolonged storage of pancake batter at ambient temperature

Seafood chowder: 2 cases. Control measure failures: inadequate cooling, inadequate reheating.

- Overseas studies:

Barbecued pork: 139 cases. Control measure failure: inadequate cooling, prolonged storage at ambient temperature.

Chicken fried rice: 14 cases. Control measure failures: inadequate cooling of cooked rice.

Vanilla sauce: 600 cases. Control measure failure: prolonged storage at ambient temperature.

Meals-on-wheels: 49 cases. Control measure failure: prolonged storage at incorrect temperatures.

2.5.7 Other *Bacillus* species

Several *Bacillus* species other than *B. cereus* have been implicated in food poisoning episodes. The species most often implicated are *B. subtilis* and *B. licheniformis*. These organisms can produce a highly heat-stable toxin which appears similar to the emetic type produced by *B. cereus*.

- Implicated foods: Meat and vegetable products which are associated with seasonings, flour and pastry, e.g. sausage rolls, meat pies and pastries, curries and various ethnic dishes with rice, and stuffed poultry.

- Symptoms: *B. subtilis*: acute onset nausea, vomiting and stomach cramps (median 2-5 hours), often with diarrhoea. *B. licheniformis*: diarrhoea is more common than vomiting (median 8 hours).

2.6 Lignin Degradation

Plant cell wall is composing of cellulose, hemicellulose and lignin. Lignin is a complex chemical compound that had played a crucial part in conducting water in plant stems. Lignin is formed through oxidation and free radical coupling of phenylpropane units, which are cross-linked to each other with a variety of different chemical bonds. The cross-link of polysaccharides by lignin had become an obstacle for water absorption to the cell wall. This will make the plant's vascular tissue to conduct water effectively because the polysaccharides component of cell wall is hydrophilic (permeable to water) while the lignin is hydrophobic. Lignin is a formidable substrate (Higuchi, 1990; Lewis and Sarkanen, 1998) thus makes it difficult to degrade. Mammalian and other animal enzymes are unable to degrade the lignin. However, some fungi and bacteria are able to biodegrade the lignin. Lignin degradation requires oxidative attack on the carbon-carbon and ether interunit bonds. Degradation of lignin will enable to gain access to the carbohydrate polymers of plant cell walls for use as carbon and energy sources. Lignolytic enzymes such as manganese peroxidase, laccase and cellobiose dehydrogenase are some example of microbial enzyme that involved in the lignin degradation.

Previous research shown that white-rot fungus *Phanerochaete chrysosporium* is enable to degrade lignin. The enzymes from white rot fungi that catalyze the initial depolymerization of lignin are extracellular and unusually nonspecific (R. Brambl and G.A. Marzluf, 2004). A constellation of oxidases, peroxidases, and hydrogen peroxide are responsible for generating highly reactive free radicals that undergo a complex series of spontaneous cleavage reactions. The nonspecific nature and extraordinary oxidation potential of these enzymes have attracted considerable interest for industrial

applications such as biological pulping of paper, fiber bleaching, and remediation of organopollutants such as pesticides, polyaromatic hydrocarbons, PCBs and various halogenated aromatics (including dioxins), certain textile dyes, TNT, and other environmentally detrimental chemicals including cyanides, azide, carbon tetrachloride, and pentachlorophenol.

2.7 Fermentation

Fermentation is the conversion of a carbohydrate such as sugar into an acid or an alcohol. Fermentation occurs naturally in many different foods given the right conditions, and humans have intentionally made use of it for many thousands of years (WiseGeek, 2008). Fermentation is also refers to any process by which large organic molecules are broken down to simpler molecules as the result of the action of microorganisms (Encyclopedia, 2008).

Fermentation is carried out in bioreactor or also known as fermenter. This process will be done either in batch, fed-batch or continuous mode. For the batch mode, the feed enter and the product is collected at the end of the process. In the fed-batch mode, the feed is entering at specific interval and the product is collected at the end of the process. However in the continuous mode, the feed is entering continuously and the product is also collected continuously. Apart than that, fermentation is carried out either in aerobic or anaerobic condition. This condition is base on the microorganisms used. For aerobic condition, oxygen is used while fermentation is carried out without oxygen in anaerobic condition.

In fermentation, either surface culture method or submerged culture method is chosen. In surface culture method the organism is allowed to grow on the surface of a liquid medium without agitation. After an appropriate incubation period the culture filtrate is separated from the cell mass and is processed to recover the desirable product. Sometimes the biomass may be reused. Examples of such fermentations are the alcohol

production, the beer production and citric acid production. This method is generally time consuming and needs large area or space (Microbiologyprocedure, 2008). In submerged culture method, the organism is grown in a liquid medium which is vigorously aerated and agitated in large tanks called fermenter. The fermenter could be either an open tank or a closed tank and may be a batch type or a continuous type and are generally made of non-corrosive type of metal or glass lined or of wood (Microbiologyprocedure, 2008).