FABRICATION OF ANTIBACTERIAL BIO COMPOSITE FROM BACTERIAL CELLULOSE AND *ARECA CATECHU* EXTRACT

KHALILURRAHMAN BIN AZIZAN

BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY)

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KHALILURRAHMAN AZIZAN BACHELOR OF CHEMICAL ENGINEERING (BIOTECHNOLOGY) 2012 UMP

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BORANG PENGESAHAN STATUS TESIS♦

JUDUL : FABRICATION OF ANTIBACTERIAL BIO COMPOSITE FROM BACTERIAL CELLULOSE AND *ARECA CATECHU* EXTRACT

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FABRICATION OF ANTIBACTERIAL BIO COMPOSITE FROM BACTERIAL CELLULOSE AND *ARECA CATECHU* EXTRACT

KHALILURRAHMAN BIN AZIZAN

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

Faculty of Chemical and Natural Resources Engineering
Universiti Malaysia Pahang

JANUARY 2012

SUPERVISOR'S DECLARATION

"I hereby declare that I have read this project report and in my opinion this project report is sufficient in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)"

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Date : 25 JANUARY 2012

STUDENT'S DECLARATION

"I hereby declare that this thesis entitled "Fabrication of antibacterial bio composite from bacterial cellulose and *Areca Catechu* extract" is the result of my own research except as cited 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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I dedicate this entire work to my family especially to my beloved parents, whose patient, support and companionship have facilitated my study, and made my life enjoyable. And not forgot to all my friends for their enduring faith and unconditional love in good times and bad.

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Last but not least, I do hope that this report will give the readers some insight as to the maze of activities associated with the fabrication of antibacterial bio composite from bacterial cellulose (BC) and *Areca catechu* extract from its planning stages until the final analysis and report.

ABSTRACT

Wound can be defined as any physical injury involving a break in the skin that usually caused by an act or accident rather than by a disease. The poor healing wound may slow down the healing process thus will be exposed to the infection of bacteria. To prevent the infection to do not further develop, it is important that the wounds be treated earlier. Therefore, the objective in this study was to produce antibacterial bio composite from bacterial cellulose (BC), starch, glycerol and areca nut extract. This bio composite, besides having the antibacterial properties that are needed for wound healing process, it also possess biodegradable capability. The 25 types of bio composite were fabricated from difference composition of bacterial cellulose (0 wt. %, 7 wt. %, 14 wt. %, 21 wt. % and 28 wt. %) and areca nut extract (0%, 25%, 50%, 75% and 100%).These films will be characterized by using phenolic content test, testing for antibacterial activity, biodegradation by using fungus, soil degradable test, water absorption test, quantitative of tannin test, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM).The antibacterial bio composite showed the presence of phenol and tannin compound by the phenolic content and quantitative of tannin test. The bio composite film that had 100% areca nut extract displayed better antibacterial properties for antimicrobial test against *E. coli* and *S. aureus*. The composite shows the degradable characteristic by the soil degradable test and test of biodegradable by incubate the film with *Aspergillus niger* strain due to the decrease of bio composite weight. For the water absorption test, the highest percentage of areca nut extract and lowest percentage of bacterial cellulose showed the optimum water uptake. From the FTIR test, the bio composite showed the presence of a carbonyl group, hydroxyl group and carbon-oxygen (C-O) bond. An addition, Scanning Electron Microscopy (SEM) was used to observe surface and cross section of the bio composite films. In order to improve the production of antibacterial film, more study should be done to investigate the effect when the film expose to the environment conditions. The fresh areca nut should be used to make sure the phenolic content in the areca nut do not degrade or decrease. Some improvement can be done by study the most suitable solvent that can be used to improve the extraction process. The combination of bacterial cellulose (BC), starch and areca nut extract can be used to produce antibacterial bio composite films which give benefit for wound healing process.

ABSTRAK

Luka ialah setiap kecederaan fizikal yang melibatkan kecederaan di kulit yang biasanya disebabkan oleh tindakan atau kemalangan dan bukan disebabkan oleh penyakit. Kaedah penyembuhan luka yang tidak betul boleh melambatkan proses penyembuhan dan terdedah kepada jangkitan bakteria. Untuk mencegah jangkitan daripada merebak, adalah penting bahawa luka akan diubati awal. Oleh sebab itu, kajian ini bertujuan untuk menghasilkan biokomposit antibakteria dari selulosa bakteria, kanji, gelatin dan ekstrak buah pinang. Bukan sahaja biokomposit ini mengandungi sifat-sifat antibakteria, malah ia juga mempunyai kebolehan untuk terurai. 25 jenis biokomposit dibuat daripada berbeza komposisi Selulosa Bakteria (0g, 7g, 14g, 21g dan 28g) dan ekstrak buah pinang (0%, 25%, 50%, 75% dan 100%).Filem-filem ini akan dikategorikan dengan menggunakan kandungan fenolik, ujian untuk aktiviti antibakteria, ujianbiodegradasi dengan mengunakan tanah dan kehadiran kulat *Aspergillus niger* sebagai agen penguraian, ujian penyerapan air, kuantitatif tanin,*Fourier Transform Infrared Spectroscopy* (FTIR)dan.*Scanning Electron Microscopy* (SEM). Biokomposit antibakteria menunjukkan kehadiran sebatian fenol dan tanin daripada ujian kandungan fenolik dan kuantitatif tanin. Filem biokomposit yang mempunyai 100% ekstrak buah pinang memaparkan ciri-ciri antibakteria yang terbaik untuk ujian antimikrob terhadap *E. Coli*dan *S. Aureus*. Dalam ujian biodegradasi menggunakan tanah dan penguraian dengan kehadiran kulat *Aspergillus niger*, biokomposit menunjukkan sifat kebolehan untuk mengurai berdasarkan kehilangan berat biokomposit Untuk ujian penyerapan air, peratus anekstrak buah pinang yang tertinggi dan peratusan selulosa bakteria yang terendah menunjukkan pengambilan air yang optimum.Dari ujian FTIR, biokompositfilemyang dihasilkan menunjukkan kehadiran kumpulan karbonil, kumpulan hidroksil dan ikatan karbon-oksigen (C-O). Sebagai tambahan, *Scanning Electron Microscopy* (SEM) digunakan untuk memerhati permukaan dan keratan rentas biokompost. Untuk meningkatkan kadar penghasilan filem anti-bakteria, lebih banyak kajian perlu dilaksanakan untuk mengkaji kesan keatas biokomposit apabila didedahkan kepada alam sekitar. Buah pinang yang segar perlu digunakan untuk memastikan kandungan fenolik dan tanin tidak berkurang. Kajian terhadap jenis-jenis pelarut yang terbaik patut dijalankan untuk meningkatkan kualiti proses pengestrakan. Gabungan selulosa bakteria, kanji dan ekstrak buah pinang boleh digunakan untuk menghasilkan filem anti-bakteria biokomposit yang memberi manfaat untuk proses penyembuhan luka.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

^{o}C	Celcius
cm	Centimeter
cm^{-1}	Per centimeter
cm^2	Centimeter square
cm^2/min	Centimeter square per min
g	Gram
g/L	Gram per liter
Gpa	Giga Pascal
h	Hour, Thickness of the film
IR	Infrared
k	Slope
Kg m^{-3}	Kilogram per meter cube
kV	Kilovolt
M	Molar
M_m	Moisture content at equilibrium
M_t	Moisture content at time *t*
mg/L	Milligram per liter
mg/ml	Milligram per milliliter
min	Minutes
mL min^{-1}	Milliliter per minutes
mm	Millimeter

nm	Nanometer
W_h	Weight of humid specimens
W_o	Initial weight of specimens
wt	Weight
w/w	Weight per weight
%	Percent

LIST OF ABBREVIATIONS

BC	Bacterial cellulose
CO_2	Carbon Dioxide
D	Diffusion coefficient
DI	Deionizer
FTIR	Fourier Transform Infrared Spectroscopy
OD	Optical Density
RH	Relative Humidity
SEM	Scanning Electron Microscope
PC	Plant Cellulose
TC	Terminal Complex
TPS	Thermoplastic Starch

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Wound can be defined as any physical injury involving a break in the skin that usually caused by an act or accident rather than by a disease. Wounds can be classified as open or closed. An open wound is a break in the skin or in a mucous membrane while closed wound involves underlying tissues without a break in the skin or a mucous membrane. The local effects of an open or closed wound may include loss of blood, interference with blood supply, destruction of tissues, nerve injury, functional disturbances, and contamination with foreign material. From the research conducted by MacKay and Miller (2003), the definition of wound healing is the body's natural process of regenerating dermal and epidermal tissue.

The healing process includes absorption of blood and serum that have seeped into the area, repair of injured cells, replacement of dead cells with scar tissue, and recovery of the body from functional disturbances. The poor healing wound may slow down the healing process thus will be exposed to the infection of bacteria. To prevent the infection for further develops, it is important that the wounds be treated earlier. The process of healing should starts immediately after an injury and may continue for months or years until the wound recovers. The cleanliness of the wound site must be maintained throughout the healing process.

There are a few herbs that have been used as wound healing. Areca nut is one of the popular traditional herbal medicines and widely cultivated throughout Thailand and South Asia. The areca nut is also used for chewing for people in some Asian countries. Karphrom et al. (2009) state that areca nuts contained hydrolysable tannin, condense tannin, some alkaloids and fats. From the research conducted by Buhler and Miranda (2000), the phenolic compounds in areca nuts were reported to have effect on anti-virus and anti-microorganisms.

According to Czaja et al. (2006), bacterial cellulose (BC) has recently attracted a great deal of attention for biomedical application such as artificial skin burn or wound healing material. To broaden the biomedical applications of bacterial cellulose (BC), various attempts have been made to produce bacterial cellulose (BC) bio composites with high functionality (Yasuda et al., 2005). Bacterial cellulose (BC)/areca nut extract bio composite is one of the candidates that have great potential applications as antibacterial bio composite. Bacterial cellulose (BC)/areca nut extract antibacterial bio composite was prepared by adding the areca nut extract in mixture of blend bacterial cellulose (BC), starch, glycerol and water followed by casting process. The antibacterial bio composite aim to restore the milieu required for skin regeneration and to protect the wound from environmental threats and penetration of bacteria. However, bio composite films dressing are better suited for small wound, since they lack absorbance and are impermeable to water vapor and gases (Jonathan and Zilberman, 2009).

1.2 PROBLEM STATEMENT

Wounds are the physical injuries that result in an opening or breaking of the skin and appropriate method for healing of wounds is essential for the restoration of disrupted anatomical continuity and disturbed functional status of the skin (Meenakshi et al., 2006). In other words, wound is a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of underlying normal tissue and may also result from a contusion, hematoma, laceration or an abrasion (Enoch and Leaper,

2005). Healing of wounds starts from the moment of injury and can continue for varying periods of time depending on the extent of wound and the process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and finally the remodeling phase (Sumitra et al., 2005). The improper techniques of wound healing will make the wound become worst. To solve this problem, the development of alternative materials for dressing the wound infection is importance.

The conventional method of wound dressing process used gauze as the protection for external bacterial infection. However this method can cause the loss of protection when the outer surface of the dressing becomes moistened by wound exudate or external fluids (Jonathan and Zilberman, 2009). Compared to gauze dressing, the bacterial cellulose (BC) based bio composite has higher water holding capacity and water vapor transmission rate thus it can maintain the moist environment at the wound/dressing surface more longer than the conventional method. Another disadvantage of conventional dressing method is the discharge rate of the drug is very rapid causing the drug retaining capability is very low. With this new technique by using bacterial cellulose (BC) as the matrix, the drug containing capability can be increased due to the strong hydrogen bond between the carbonyl group from cellulose and hydroxyl group from the phenolic content in areca nut extract. This study will focus on the production of antibacterial bio composite from bacterial cellulose (BC) with areca nut extract. Areca nut is selected for the antibacterial materials based on the phenolic compound content in the nut. These combinations can be used in the application of wound healing.

1.3 RESEARCH OBJECTIVE

The main objective in this study is to produce antibacterial bio composite from bacterial cellulose (BC) and areca nut extract.

1.4 SCOPE

1. To fabricate the antibacterial bio composite from the bacterial cellulose (BC)(0wt. %, 7wt. %, 14wt. %, 21wt. % and 28wt. %) and areca nut extract (0%, 25%, 50%, 75% and 100%) by using casting method.

2. To characterize the characteristic of bio composite produced by using Water Absorption test, Phenolic content test, quantitative of tannin test, Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), biodegradable by using *Aspergillus niger*, soil degradable test and testing for antibacterial activity by using Disc Diffusion assay.

1.5 RATIONAL AND SIGNIFICANCE

Being in line with the advanced lifestyles, the trends of pharmaceutical market in the world today also aware that there is a growing demand for instant remedies that easy to be used. People are exposed to get wound for each single time and they need an easy method to heal with a simple technique. To seize this opportunity, a compound or substance that has an ability to kill or slow down the growth of bacteria at specific wound is tended to be produced. Areca nut, is a seed of Areca palm (*Areca catechu*), has a potential to become antibacterial material because of the phenolic content contains in it (Buhler and Miranda, 2000). With a new invention of combination of starch and bacterial cellulose (BC) as biodegradable polymer with the antibacterial compound, it would give such an impressive market value product. Wound healing bio composite is not just good for pharmaceutical purposes, but also for the environment. This is due to its ability which can degrade by time.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Wound healing process needs to be take care from infection which can slow down the healing process. Thus, the production of antibacterial bio composite from bacterial cellulose (BC) and areca nut extract will be used as an alternative wound dressing to prevent this situation. Areca nut has been used in many places as the medicine because of its phenolic antioxidant attribute. A review is performed to identify studies that relevant to this study on production of antibacterial bio composite from bacterial cellulose (BC) and areca nut extract. This research is basically about the findings of an effective composition of bacterial cellulose (BC) and areca nut extract to produce antibacterial bio composite for wound healing process. Therefore this chapter provides reviews about the main component of this study and characterization of bio composite.

2.2 BACTERIAL CELLULOSE (BC)

Cellulose is the most abundant biopolymer on earth, recognized as the major component of plant biomass, but also a representative of microbial extracellular polymers. An efficient producer of cellulose is acetic acid bacteria *Acetobacter xylinum*. Several different techniques for bacterial cellulose (BC) production have been reported such as stationary culture, agitated culture, cultivation in the horizontal fermenters or cultivation in the internal-loop airlift reactors. Stationary cultivation methods produce pellicles of bacterial cellulose (BC) that being formed on the surface of the static culture (Son et al.,

2003). Table 2.1 below shows the comparison between the results of various cultural conditions used by different researchers.

Table 2.1: The comparison between the results of various cultural conditions used by different researchers.

Volume (L)	Yield (g/L)	System	Temp. (°C)	pH	Time (h)	References
-	9.7	Shaking	-	-	7	Tsuchida et al. (1997)
30	20	Shaking	30	5	42	Kouda et al. (1998)
2	15	Shaking	30	5/5	50	Hwang et al. (1999)
Tubes	3	Static	30	5/6-7/5	4 weeks	Ishihara et al. (2002)
0.075	16.4	Shaking	30	5/6	192	Son et al. (2002)
0.61	21	Shaking	30	5	50	Naritomi et al. (2002)
0.1	12.8	Shaking	30	5	72	Bae et al. (2004)
0.03	-	Static	28	6	168	Keshk et al. (2005)

Source: Pourramezan et al. (2009)

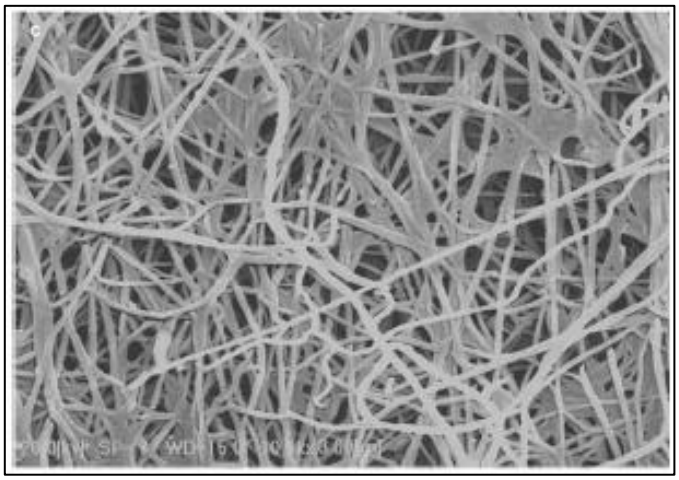

Figure 2.1: Scanning electron microscopy images of BC membrane from static culture of *Acetobacter xylinum.*

Source: Kim et al. (2010)

The bio composite produced from bacterial cellulose (BC) and starch present important advantages such as high availability, biodegradability, specific strength and modulus, sound attenuation, comparatively processing ability and low cost, energy consumption, density due to their flexibility and non-abrasive nature (Martins et al, 2009). The other researchers (Wan et al., 2007) also state that the bacterial cellulose produced by *Acetobacter xylinum* has unique, properties including high water-holding capacity, crystallinity, hydrophilicity, higher tensile strength and a pure ultra-fine fibre network structure. Based on research from Yano et al. (2005), the bacterial cellulose (BC) micro fibrils have a density of 1600 kg m−3, Young's modulus of 138 GPa, and tensile strength of at least 2 GPa.

Extensive research on bacterial cellulose (BC) revealed that it is chemically identical to plant cellulose (PC), but its macromolecular structure and properties are different. The molecular formula of bacterial cellulose (BC) $(C_6H_{10}O_5)_n$ is the same as the plant cellulose, but their physical and chemical features are different. Bacterial cellulose (BC) is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization, crystallinity index, tensile strength and water holding capacity compared with plant cellulose, making it more suitable as raw material for producing high fidelity acoustic speakers, high quality paper and dessert foods. Ongoing research of bacterial cellulose (BC) products includes a wide range of biomedical applications such as in treatment of chronic wounds and burns as temporary coverage, artificial cardiovascular tissues, and guided regeneration of bone, cartilage and nerve. Figure 2.2 shows the structure of bacterial cellulose (BC).

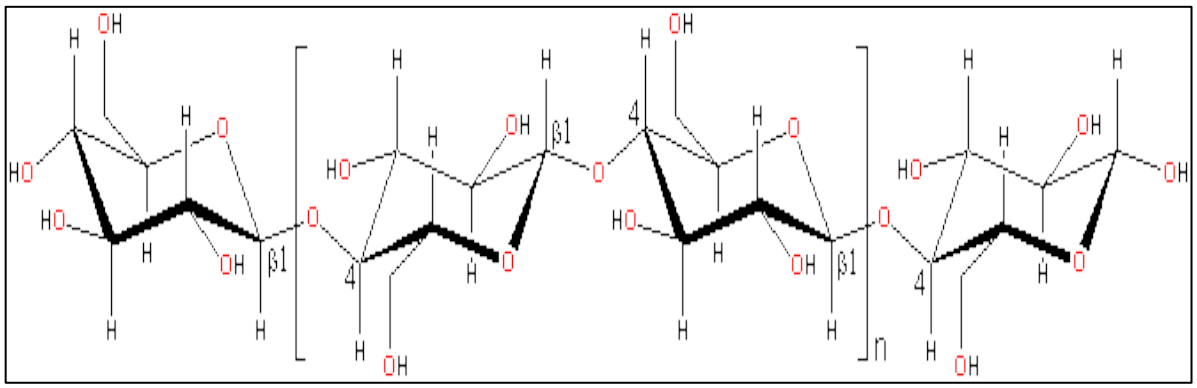

Figure 2.2: Bacterial cellulose structure

Source: http://www.lsbu.ac.uk/water/hycel.html (retrieve at 17 September 2011)

2.3 *ACETOBACTER XYLINUM*

Acetobacter xylinum is a gram negative bacterium and have used to synthesis of cellulose. According to Chawla et al. (2009), *Acetobacter xylinum* converts various carbon compounds, such as hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids, into cellulose. The conversion is usually about 50% efficiency. Other gram-negative species like *Agrobacterium, Achromobacter, Aerobacter, Sarcina, Azotobacter, Rhizobium, Pseudomonas, Salmonella and Alcaligenes*can also produce bacterial cellulose. Based on research conducted by Ross et al. (1991), *Acetobacter xylinum* is most effective producers of cellulose. *Acetobacter xylinum* was cultured in coconut-water supplemented with 5.0% sucrose, 0.5% ammonium sulfate and 1.0% acetic acid. The culture media was incubated statically at 30°C for 7 days (Saibuatong and Phisalaphong, 2010). Based on research done by Krystynowic et al. (2005), the optimum temperature for *Acetobacter xylinum* growth is 25–30°C, and pH ranges from 5.4 to 6.2.

According to Bielecki et al. (2005), *Acetobacter xylinum* has been applied as a model microorganism for basic and applied studies on cellulose. It is an ability to produce relatively high levels of polymer from a wide range of carbon and nitrogen sources. It is a

rod-shaped, aerobic, gram-negative bacterium that produces cellulose in the form of interwoven extracellular ribbons as part of primary metabolite. This bacterium grows and produces cellulose from a wide variety of substrates and is devoid of cellulose activity.

Klemma et al. (2001) has mentioned about the production of bacterial cellulose (BC) from *Acetobacter xylinum* in their research. The cellulose was constituted between the outer and the cytoplasma membrane. The cellulose was formed by synthesizing complexes or terminal complexes (TC) that are linearly arranged, and in association with pores at the surface of the bacterium. The glucan chain from the terminal complexes will elongates, where 6-8 glucan chain will be combined together. This combined glucan chain, as known as fibril, will elongates further as micro fibril, resembles as ribbon like structure. Multiple micro fibril will eventually be interwoven together, foaming matrix like constituents of pellicle, which is the bacterial cellulose (BC) membrane. Figure 2.3 illustrated the formation of bacterial cellulose (BC).

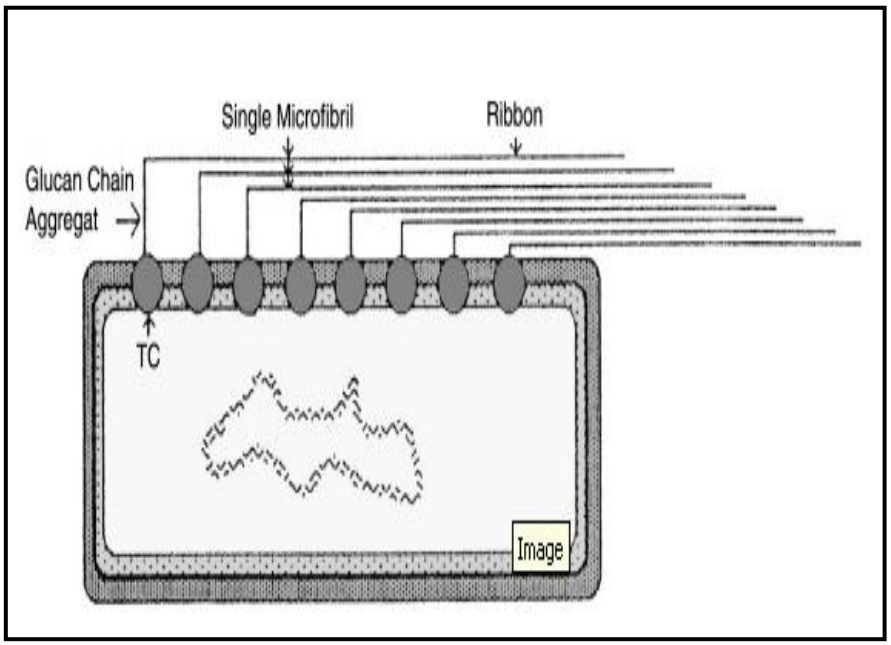

Figure 2.3: Formation of bacterial cellulose.

Source: Klemma et al. (2001)

2.4 STARCH

Starch has drawn a lot of attention in the preparation of bio composite. It is abundantly produced by photosynthetic plants and more over it is renewable and cheap. The addition of starch to synthetic plastics is known to enhance the biodegradability. Owing to its complete biodegradability, low cost and renewability, starch is considered as a promising candidate for developing sustainable materials (Lu et al., 2009). Figure 2.4 shows the structure of starch. Starch is usually utilized in the form of granules, and is actually formed by one branched and one linear polymer. Amylose, the linear polymer, comprises approximately 20% w/w of starch, while Amylopectin, the branched polymer, constitutes the remainder. Natural filler materials may be incorporated into synthetic plastic matrices as a rapid biodegradable component.

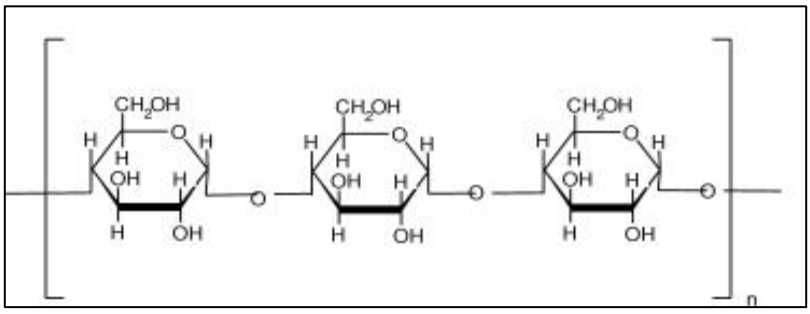

Figure 2.4: Structure of starch

Source: Lukasiewicz et al. (2007)

Starch can also be used in its gelatinized form (Verhooght et al. 1995), where if heating the starch in the presence of water during extrusion or injection moldings can cause the formation of a thermoplastic material. Heating starch above its glass transition temperature, will breaks its molecular structure and allowing further bonding. Glycerol is often used as a plasticizer in starch blends to increase softness. Starch granules that have been plasticized with water and glycerol are referred to as plasticized starches (Martins et al., 2009).

2.5 *ARECA CATECHU*

Areca nut or Betel nut (*Areca catechu*), is the seed of fruit of Areca Palm, or is known as Pinang in Malay language. According to Xing et al. (2010), Areca Palm is widely grown and consumed in some places including India, Southeast Asia, East Africa and New Guinea. Recent evidence suggests that areca nut serve as an important and popular cultural activity in many Asians and Oceanic countries, mainly as chewing material anthelmintic and also known as kompo-traditional medicine (Chung et. al., 1998). One of the main usages of Areca nut for a traditional medicinal purpose is as digestive medicine that can promotes the secretion of saliva. Figure 2.5 show the Areca nut with different shapes. The extracts yield of oval shape Areca nuts is higher than round shape Areca nuts (Karphrom et al., 2009).

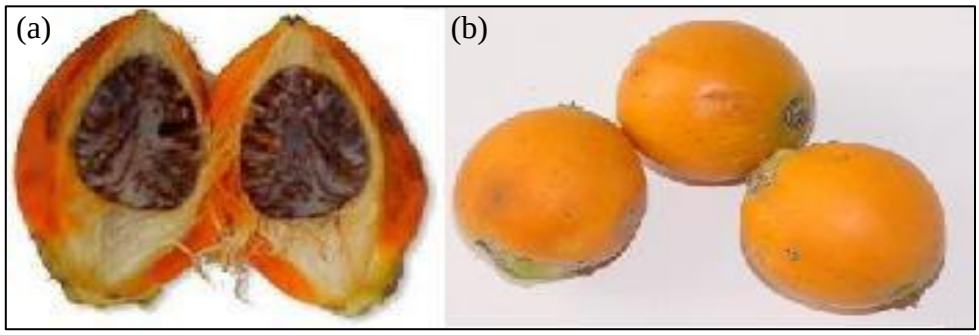

Figure 2.5: The Areca nut with oval shape (a) and round shape (b)

Source: http://www.pnm.gov.my/sirihpinang/sp-pinang.htm (retrieve at 21 October 2011)

Areca nut has been the subject of numerous studies, where the nut itself was expected to contain active compounds, such as alkaloids, tannins, flavonoids and fatty acid. Areca nut contains 50% - 60% sugars, 15% lipids, 15% condensed tannins, polyphenolics and 0.2%—0.3% alkaloids based from the research conducted by Wangcharoen et al. (2007). It has been found that Areca nut can alter the modulation of widely used phenolic

antioxidant substance, which is 2(3)-tert-buty-l4-hydroxy anisole (BHA) that can be effect on carcinogenesis.

From the entire compound that comprised in areca nut, the polyphenolics compound is the one that will be focused. Polyphenolics is a secondary metabolites involved in the chemical defense against plant diseases and plant interference. Ferrazzano et al. (2011) state that several thousand of plant polyphenols are known, encompassing a wide variety of molecules that contain at least one aromatic ring with one or more hydroxyl groups in addition to other substituents. These compounds possess the quality in biological property, which as the antioxidant (Bhattacharya et. al., 2010), anticancer (Borchardt et al., 2009), and anti-inflammatory (Bowden et. al., 1999) effects.

2.6 BIOCOMPOSITE

Biocomposite is materials that consist of two or more distinct constituent to obtain complex chemical, mechanical and biological properties. Bio composite is built up by natural cellulose fibers as reinforcement and starch or biopolymer as the natural matrix. In this research, the natural cellulose fibers used are bacterial cellulose (BC). Bio composites also called as green composites. Under specific conditions, the starch can be converted into thermoplastic starch (TPS). However, starch is not truly thermoplastic as other synthetic polymers. Thus, in order to improvise the properties of starch, it can be blended with bacterial cellulose (BC) fibers.

This bio composite is designed to be completely biodegradable. Microorganisms are able to consume these materials in their entirety, eventually leaving carbon dioxide and water as by-products. There are certain ingredients that must be present in order for biodegradation to occur. Most importantly, the active microorganisms (fungi, bacteria, actinomycetes, etc.) must exist in the disposal site. Huang et al. (1990) state that the appropriate degradation temperature wasusually falls between 20 to 60^{o}C. The disposal site must be in the presence of oxygen, moisture, and mineral nutrients, while the site pH must be neutral or slightly acidic (5 to 8). The microbes digest the starch, leaving behind a

porous, sponge like structure with a high interfacial area, and low structural strength. When the starch component has been depleted, the bio composite begins to be degraded by an enzymatic attack. Each reaction results in the scission of a molecule, slowly reducing the weight of the bio composite until the entire material has been digested (Kolybaba et al., 2003).

Many biopolymers are designed to be discarding in landfills, composts, or soil. The materials will be broken down by the present of microorganisms. The biodegradable of bio composite help the environment by reducing waste issues. There are two main reasons for using biodegradable materials which are the growing problem of waste resulting in the shortage of landfill availability and the need for the environmentally responsible use of resources (Mohanty, 2001).

2.7 *ASPERGILLUS NIGER*

The genus *Aspergillus* is a group of mould-like fungi. It was first described by Micheli in 1729, and so named by him because of its resemblance to the aspergillium, or brush used for sprinkling holy water (*aspergere L.*= sprinkle). The other species of genus *Aspergillus* are *A. fumigatus, A. nidulans, A. niger, A. clavatus,* and *A. versicolor. A. niger* is one of the most common species of the genus *Aspergillus*. It has been identified on decomposing vegetable matter, in proprietary hop manures, in various cereals, hay, straw and in rotting wood. It also occurs as a common laboratory contaminant and as a saprophyte or secondary invader in a variety of pulmonary diseases.

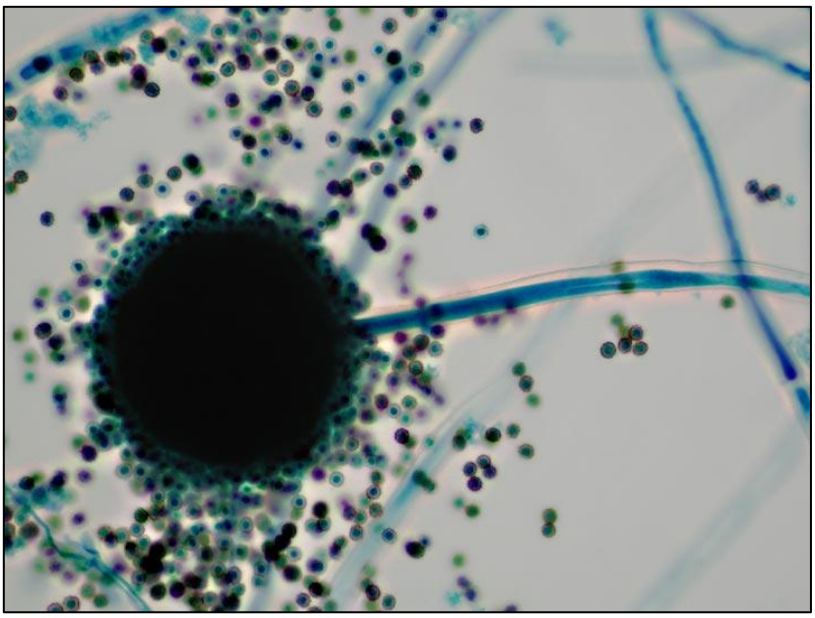

Figure 2.6: *Aspergillus niger* in slide culture.

Source:
http://labmed.ucsf.edu/education/residency/fung_morph/fungal_site/asperpage.html
(retrieve on 26 November 2011)

Microorganisms such as bacteria, fungi and actinomycetes are involved in the degradation of both natural and synthetic plastics (Gu et al., 2000). However, according to (Kim and Rhee, 2003), the growth of fungal biomass is faster compared to bacteria. Also fungi can survive in the harsh environments with low nutrient availability, low pH and low moisture content. Several analytical methods have been used to test the biodegradability of composite by using fungi which includes visual observation, changes in molar mass, weight loss measurement, CO_2 evolution and clear zone formation (Pramila and Ramesh, 2011). Based on the research conducted by Kazmarek and Bajer (2007), the additives added in the composite may enhance the physical and chemical degradation. The mixing of small amount of cellulose can bring some changes in the composite properties and lead to its microbial degradation.

2.8 BIOCOMPOSITES CHARACTERIZATION

2.8.1 Moisture Absorption Experiment

According to research done by Wan et al. (2009), the moisture absorption measurements were performed under 75% RH (relative humidity) at 25^{o}C. All specimens for moisture absorption experiments with dimensions of 30 mm by 10 mm were cut from composite sheets. Prior to the absorption experiments, all specimens were thoroughly washed and then dried until a constant weight was attained. The moisture uptake at any time points as a result of moisture absorption was determined by equation 2.1.

$$\text{Water absorption} = \frac{W_h - W_o}{W_o} \times 100 \qquad (2.1)$$

Where W_h and W_0 denote, respectively as the weight of humid specimens and the original dry value (the initial weight of materials prior to exposure to moisture absorption).

Figure 2.7 shows the moisture uptake of the bacterial cellulose (BC)/starch composites with varying fibre loading and the pure starch as a function of time. Note that the moisture uptake of the composites as well as the pure starch increases linearly in the initial stage of the absorption process, then the increasing rate slows down, and finally leads to a plateau, corresponding to the water uptake at equilibrium (Wan et al., 2009).

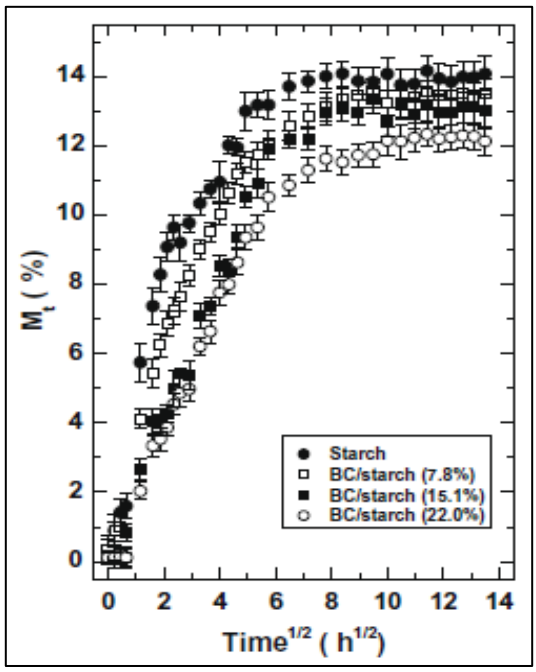

Figure 2.7: Moisture absorption curves of BC/starch bio composites with different BC contents.

Source: Martins et al. (2009)

Based on other researcher (Martins et al., 2009), the moisture absorption was slightly reduced by the incorporation of bacterial cellulose fibers which was ascribed to the facts that starch was more hydrophilic than cellulose.

2.8.2 Scanning Electron Microscopy (SEM)

A scanning electron microscope (SEM) is a type of electron microscope that images a sample by scanning it with a high-energy beam of electrons in a raster scan pattern. The sample was frozen with liquid nitrogen and immediately; the sample was snapped; vacuum dried and sputter with gold (Saibuatong and Phisalaphong, 2010). The electrons interact with the atoms that make up the sample producing signals that contain information about

the sample's surface topography, composition, and other properties such as electrical conductivity.

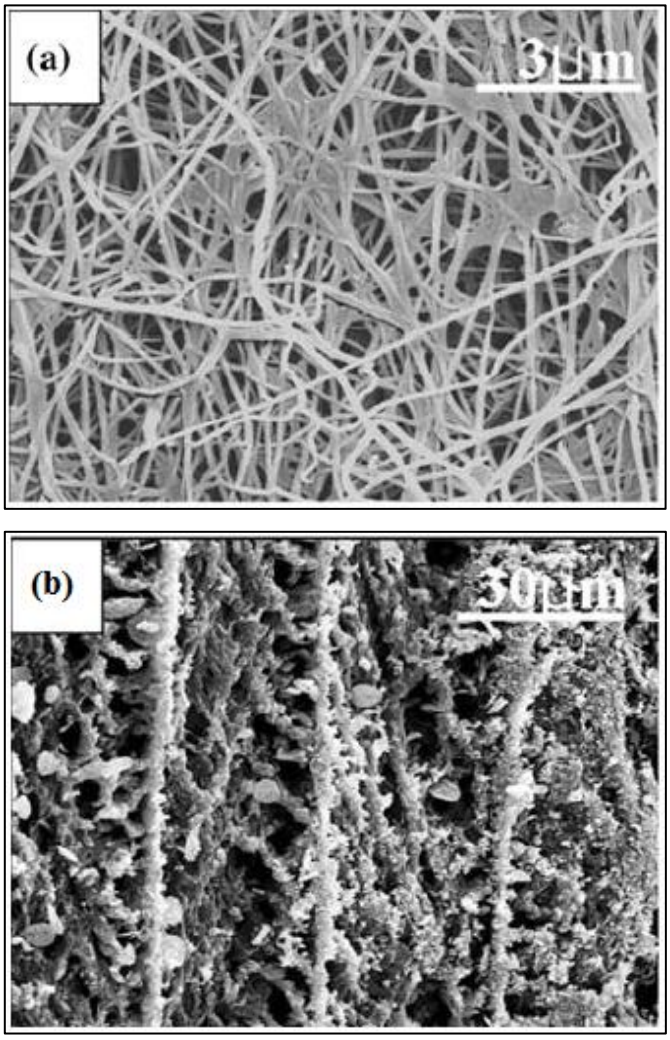

Figure 2.8: SEM of general structure of original bacterial cellulose (a) surface image (b) cross section image.

Source: (Kim et al., 2010)

Figure 2.8 shows the general structure of bacterial cellulose (BC) taken from scanning electron microscopy. Kim et al. (2010) state that bacterial cellulose (BC) ribbon-

shaped fibrils can be observed on the surface. The mean diameter of these fibrils is about 100 nm, as indicated in Figure 2.8 (a). From cross sectional images (Figure 2.8 (b)) these fibrils assemble together forming porous structure with high aspect ratio. According to another researcher (Wan et al., 2009), the bacterial cellulose (BC)/starch composites show a layered structure which displayed in Figure 2.9. The layered structure is the characteristic for bacterial cellulose (BC) network as a difference in the cellulose network structure that usually observed between the upper surface layer (exposed to air) and the lower layer (exposed to culture medium during cultivation).

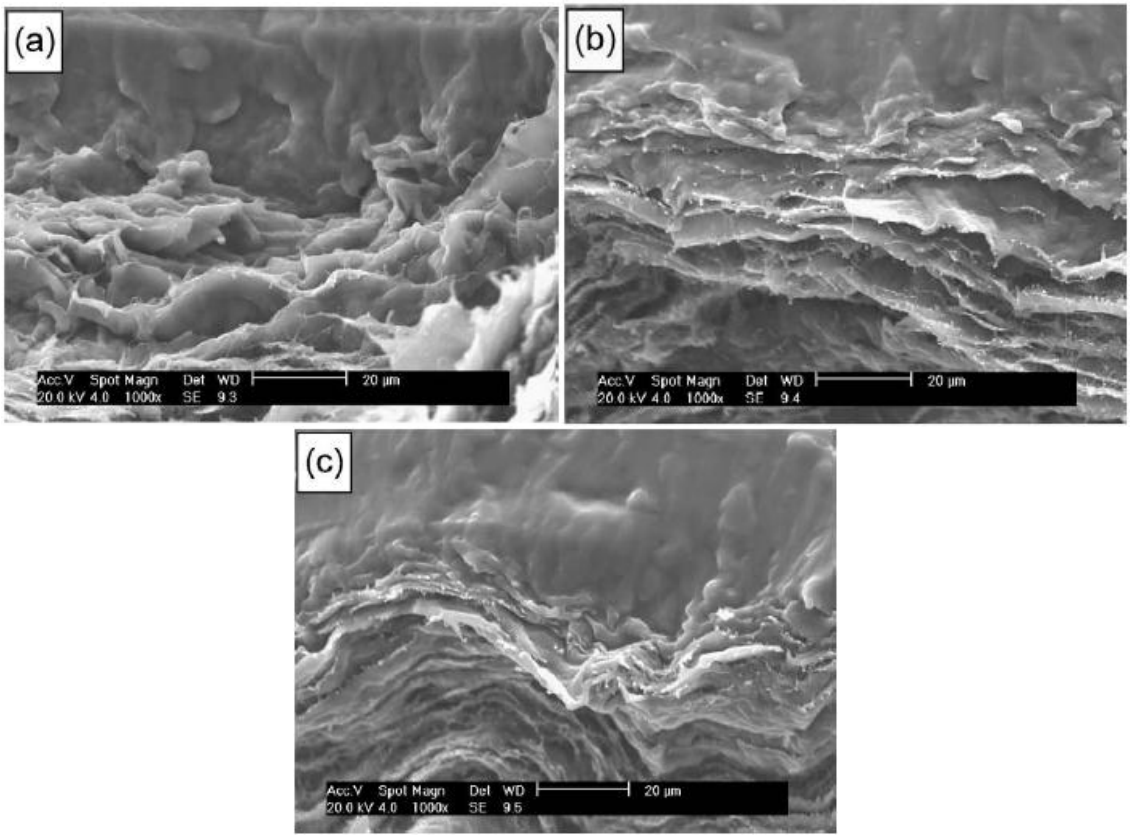

Figure 2.9: Scanning electron micrographs of tensile fracture surfaces of BC/starch biocomposites with different BC contents (a) 7.8 wt.%, (b) 15.1 wt.%, and (c) 22.0 wt. %.

Source: Wan et al. (2009)

2.8.3 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform-Infrared Spectroscopy (FTIR) is an analytical technique used to analyzing organic materials and certain inorganic materials. Lee et al. (1994) state that FTIR spectroscopy has often been utilized as a useful tool in determining specific information about chemical bonding and molecular structures that exist in a material. Chemical bonds vibrate at characteristic frequencies, and when exposed to infrared radiation, they absorb the radiation at frequencies that match with their vibration modes. The main operation's principle of FTIR is by measuring the radiation absorption as a function of frequency produces a spectrum that can be used to identify functional groups and compounds. Phisalaphong and Jatupaiboon, (2008) state that the FTIR spectra of the membranes were measured at wave numbers ranging from 2800 to 1200 cm with a Nicolet (United States) SX-170 FTIR spectrometer.

When two or more substances are mixed by physical blends, the chemical interactions are reflected by changes in the characteristic of spectral peaks. Figure 2.10 shows the Fourier Transform Infrared Spectroscopy (FTIR) diagram of bacterial cellulose. In case of pure bacterial cellulose (BC), a broad band at 3,326 cm^{-1} is attributed to O–H stretching vibration. Band at 2,896 cm^{-1} represents the aliphatic C–H stretching vibration. A sharp and steep band observed at 1,031 cm^{-1} is due to the presence of C–O–C stretching vibrations.

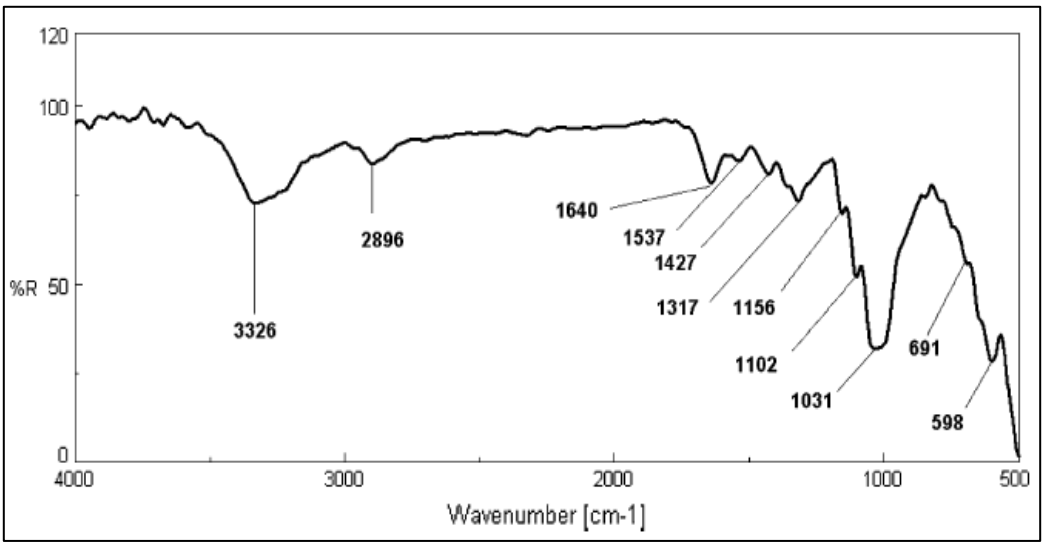

Figure 2.10: Fourier Transform Infrared Spectroscopy (FTIR) diagram of bacterial cellulose (BC).

Source: Guzun et al. (2011).

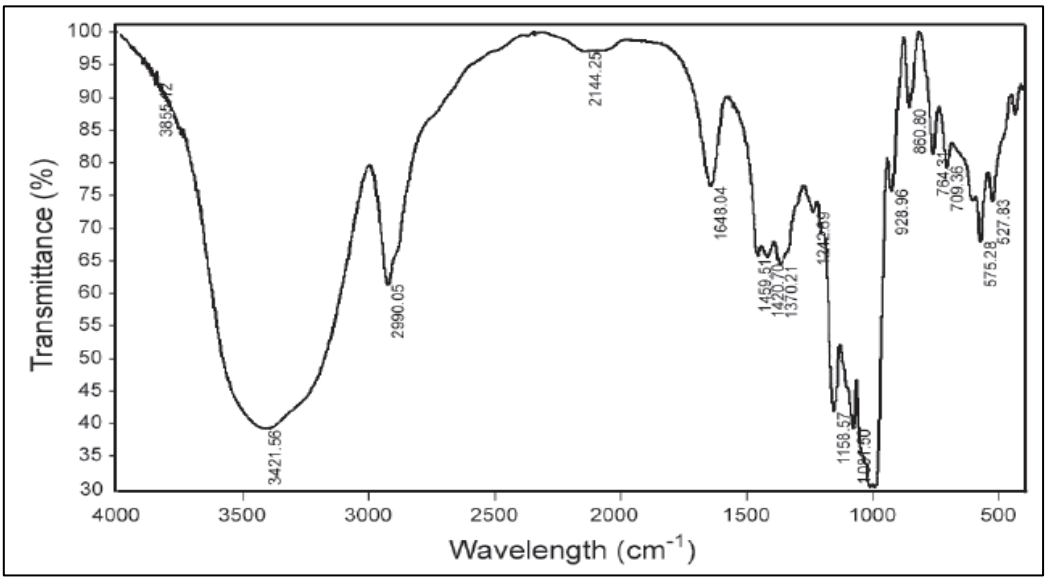

Figure 2.11: Fourier Transform Infrared Spectroscopy (FTIR) diagram of pure starch.

Source: Nazarzadeh et al. (2011)

According to Guzun et al, (2011), the bio composite samples developed by the supplement of areca nut extract present a peak shift and an occurrence of new peak from 3200 to 3500 cm^{-1} due to incident of the intermolecular interaction of bacterial cellulose (BC) and the hydroxyl group of areca nut extract. The FTIR spectrum of pure starch is shown in Figure 2.11. The characteristic broad band for O-H group (in the presence of H-band) of starch appears at 3421 cm-1. A peak around 2930 cm-1 is attributed to an asymmetrically stretching vibration of C-H band in pyranoid ring. The several absorption bands between 828 and 1158 cm^{-1} are attributed to the contribution of various functional groups, such as C-O and C-O-C (Nazarzadeh et al., 2011).

2.8.4 Soil Burial Degradation Experiments

According to Mostafa et al. (2010), biodegradation can be defined as the degradation process caused by biological activity; particularly by enzyme action leading to significant changes in the material's chemical structure. The biodegradability of plastics is dependent on the chemical structure of the material. Albertsson and Karlsson (1995) claim that biodegradation process of the starch-based polymer is associated with the loss of mechanical properties, increased permeability and greater surface/ volume ratio. The starch particles can swell and cause disruption of the polymer surface if the degradation takes place in aqueous environments. Enzymatic breakdown of the starch particles will provide an intermediate carbon source for the invading microorganisms to the degradable process.

Soil burial degradation experiments were carried out at an ambient temperature under moisture controlled conditions. The pieces of bio composite were placed in a series of boxes containing moisturized soil. The specimens (30 x 10 mm) were buried 100 mm beneath the surface of soil which was regularly moistened with distilled water (Wan et al., 2009). The test was conducted every 6 days up to 24 days of soil burial. The specimens were removed, rinsed with distilled water and dried in an oven at 60°C for 4 hours before weighed. The specimens were weighed on the analytical balance in order to determine the average weight loss:

$$\text{Weight loss} = \frac{W_o - W_t}{W_t} \times 100 \quad (2.2)$$

Where W_o is the initial mass and W_t is the remaining mass at any given time, t.

The microorganisms will degrade cellulose to glucose and the final product from aerobic biodegradation was CO_2 and water. The bio composite that composed of bacterial cellulose had a microfiber and will take prolong degradable time. It was because of the interspace forming between the fiber of bacterial cellulose and starch. The bacterial cellulose formed the crystalline regions that were difficult to degrade (Alvarez et al., 2006).

The other researcher also studies on the biodegradation behavior of the bio composite material. Figure 2.11 shows an approximately linear relation between degradation time and different composition of bio composite that has been done by Wan et al, (2009). The average degradation rate was about 1%/day and 0.9%/day, respectively, for the starch and the composite. It was observed that the weight loss of the bacterial cellulose (BC)/starch composite is lower than the pure starch at any given time points

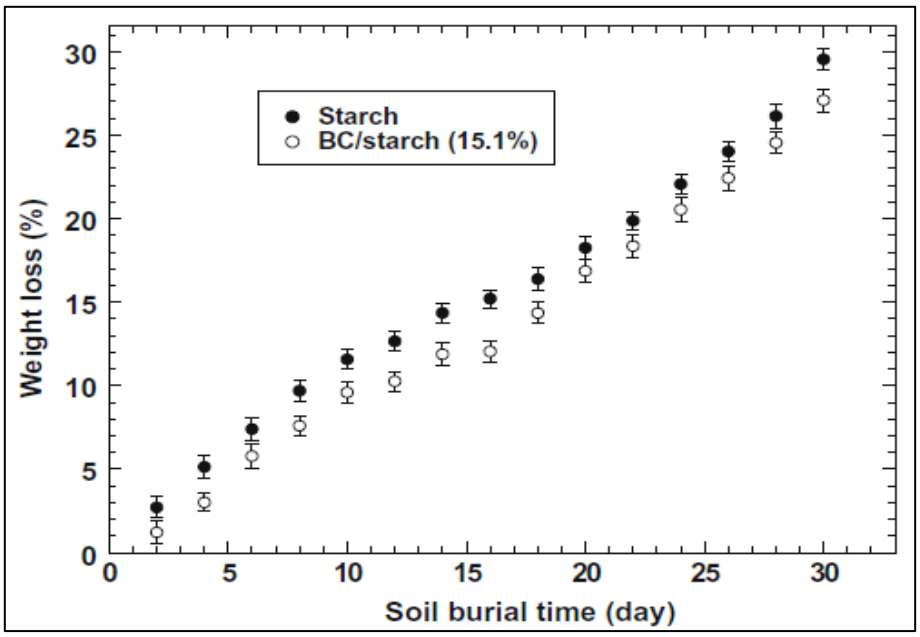

Figure 2.12: Weight loss of BC/starch biocomposite and starch as a function of soil burial time.

Source: Wan et al. (2009)

2.8.5 Antibacterial Properties

2.8.5.1 Disc Diffusion assay

Disc Diffusion Assay method is the simplest way of determining the activity of bacteria against the antibacterial agent by placing the bio composite on the agar plate to allow the antibacterial agent to diffuse into the agar medium. The antibacterial activities of bio composite will be evaluated using two different bacteria; *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). From the research conducted by Tebai et al. (2009), it stated that areca nut (*Areca catechu*) had tannin substance as an antibacterial which can destructs bacteria toxin substance and bacteria cell protein. Phenol compound in areca has an antibacterial compound that capable of destruct cell wall, cell cytoplast membrane and denature bacterial protein included *Staphylococcus aureus* (*S. aureus*).

Table 2.2: The minimal inhibitory concentration (MIC) of Areca nut extracts on some food borne pathogenic bacteria.

Fruit shape	Maturity	Solvent	MIC (mg/ml)			
			B.cereus	*S.aureus*	*E.coli*	*S.typhimurium*
Round	Mature	Water	0.78	0.78	1.56	1.56
Round	Immature	Water	0.78	0.78	1.56	1.56
Oval	Mature	Water	0.78	0.78	0.78	1.56
Oval	Immature	Water	1.56	1.56	1.56	3.13

Source: Karphrom et al. (2009)

Table 2.2 tabulates the minimal inhibitory concentration of areca nut extracts on the different type of bacteria. According to Karphrom et al. (2009), the best anti-bacterial activities of the areca nuts one from water extraction of the round shaped and mature fruits, with MIC at 0.78 mg/ml for all four test organisms.

2.8.5.2 Phenolic Content Analysis

In recent years, a large number of studies have been done on the antifungal activity of phenolic compounds of natural origin. Phenolic compounds have diverse defensive functions in plants, such as cell wall strengthening and repair, and also antimicrobial and antifungal activities. In order to quantify the amount of total phenolic compounds contained in the bio composite produced, the Folin-Ciocalteu method will be used in this study. Based on the research conducted by Ferrazzano et al. (2011), phenolic constituents occur on the surface of plants or in the cytoplasmic fraction of the epidermal cells, where they act as a deterrent to pathogens. Phenol is one of the major compound contains in areca nut. Wetwitayaklung et al, (2006) state that, areca nuts contain alkaloids, tannins, polyphenols, sugars and lipids that has anthelmintic, antifungal, antibacterial, anti-inflammatory and antioxidant activities.

2.8.5.3 Quantitative of Tannins

Areca catechu has traditionally been used as herbal medicines in many countries. Its seed is chewed as areca quid and used as medicines. According to Wetwitayaklung et al, (2006), the areca nut contains 50-60% sugars, 15% lipid, 15% condensed tannins, polyphenolics and 0.2-0.5% alkaloids. Tannins are water-soluble polyphenols that are commonly found in higher herbaceous and woody plant. They can be classified into two categories: hydrolysable and non-hydrolysable (Akiyama et al., 2001). Tannin was found to be inhibitory to the growth of intestinal bacteria such as *Bacteroidesfragilis*, *Clostridium perfringes*, *Escherichia coli* and *Enterobacter cloacae* amongst others. Chung et al, (1998), reported that the inhibitory effect of tannin on the growth of intestinal bacteria mat be caused by its strong iron-binding capacity. The method used to determine the tannin compound is the Lowenthal Permanganate Titration method (Kumazawa et al., 2002). Tannins have a major impact on animal nutrition because of their ability to form complexes with numerous types of molecules. Starch has the ability to form hydrophobic cavities that allow inclusion complexes with tannins and many other lipophyllic molecules while cellulose has a direct surface interaction with tannins.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

In this chapter, all the methodology used to make the antibacterial bio composite from bacterial cellulose (BC) and *Areca catechu* extract was presented one by one. The first method was the production of bacterial cellulose from fermentation of *Acetobacter xylinum* in coconut water mediums. The next method was the fabrication the mixture of bacterial cellulose, starch, glycerol and areca nut extract to get the antibacterial bio composite plastic. Finally, the films produce were characterized by phenolic content test, testing for antibacterial activity, biodegradation by using fungus, soil degradable test, water absorption test, quantitative of tannin test, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM).

3.2 MATERIAL

The stock culture of *Acetobacter xylinum* and *Aspergillus niger* was supplied by Malaysia Agriculture Research and Development Institute (MARDI), Serdang, Selangor. The bacterial cellulose was obtained from the fermentation of *Acetobacter xylinum* in the coconut water. All the reagents such as Starch, Sodium Hydroxide (NaOH), ethanol, Glycerol, Ammonium Sulfate ($(NH_4)_2SO_4$), and Folin Ciocalteu Reagent were purchased from Sigma, Chemical Co. St. Louis. The *Areca catechu* was collected from Kg Tanjung Sedili, Kota Tinggi, Johor. The soils for the soil burial test were taken from the field nearby the university campus.

3.3 EXPERIMENTAL PROCEDURE

Figure 3.1 show the overall procedure done to fabricate film sample. Firstly, the culture media was prepared by mixing coconut water, ammonium sulphate, acetic acid and glucose. The culture media was used to inoculate the stock medium. In the next step, several film samples were produced by using the casting method. Bacterial cellulose produced from the fermentation of *Acetobacter xylinum* was mixed with starch, water and glycerol and various percentage of Areca catechu extract. Finally, the films produced were analyzed and characterized by using phenolic content test, testing for antibacterial activity, biodegradation by using fungus, soil degradable test, water absorption test, quantitative of tannin test, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM).

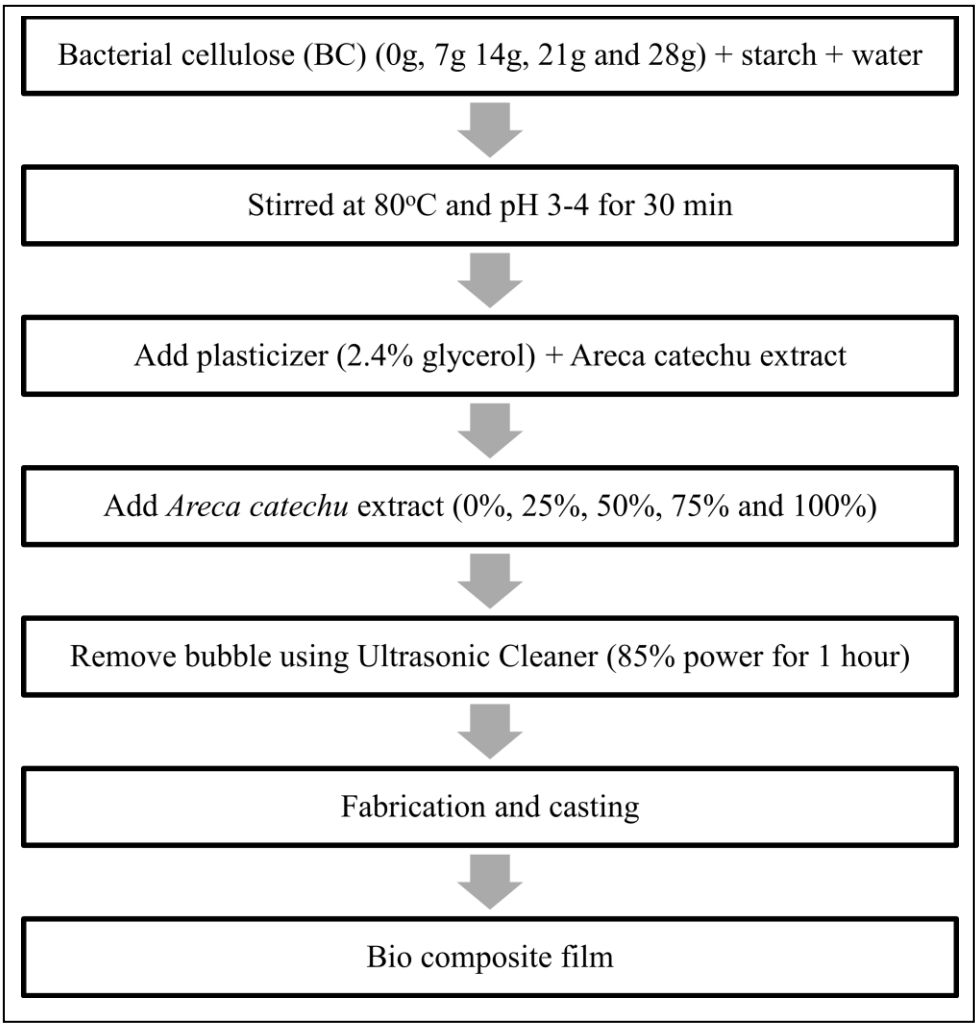

Figure 3.1: Experimental procedure for production of bio composite film

3.3.1 BACTERIAL CELLULOSE (BC) PRODUCTION

Bacterial cellulose (BC) was prepared by ferment the *Acetobacter xylinum* in coconut water containing 8.0% sucrose, 0.5% ammonium sulfate ($(NH_4)_2SO_4$) and 1.0 % acetic acid. The 100 mL of a stock culture was then inoculated into 1000 mL of medium and incubated at 30°C for 7 days in a static culture. Phisalaphong and Jatupaiboon (2008) claim that the white membrane (bacterial cellulose) was formed at the surface of the medium after 7 days. The obtained gel-like bacterial cellulose (BC) was purified by immersion in a 0.5 M aqueous solution of Sodium Hydroxide for 15 min. The bacterial

cellulose (BC) pellicle was washed with deionize water several times. Next, the starch was dissolved in distilled water at concentration of 6 g/ 100 mL by heating the mixtures on hot plate and stirred the mixture until it gelatinized at temperature of 80°C for about 30 minutes. From the research conducted by Wan et al. (2009), it showed that the optimum pH of the resulting solutions was kept at 3-4.

3.3.2 PREPARATION OF ARECA NUT EXTRACT

The fresh areca nuts taken from Kg Tanjung Sedili were washed with distilled water. Based on the research conducted by Karphrom et al. (2009), the nuts were sliced in small pieces and dried at 60^{o}C for 3 days. The dried nuts were blended to obtain the fine powder in the blender. Then, the powders were extracted by mixing 75g of areca powder to 150g of water. The mixture was shaked in a bath shaker at 80°C and 100 rpm for 2 hours. The aqueous extract of areca nut was filtered by using filter paper. The clear supernatant was dried in an oven at 60°C for 5 days. The dried areca nut extract was kept in a desiccator until the experiment began.

3.3.3 FABRICATION OF BACTERIAL CELLULOSE AND ARECA NUTS EXTRACT BIO COMPOSITE

The solutions were prepared by mixing 100 mL of the starch solution (6 g/ 100 mL) and bacterial cellulose (0, 7, 14, 21 and 28 wt%). The solutions were mixed in the mixer until it becomes gelatinized. Glycerol was added as 2.4% (w/w) of the total solid weight in the blend solutions. After that, the various areca nut extracts (0%, 25%, 50%, 75% and 100%) were added to the mixture of bacterial cellulose (BC), starch and glycerol. The resulting solutions were put in the Ultrasonic Cleaner at 85% power for one hour. Then, the solutions were poured into mold and dried for 4 hours. Based on research conducted by Phisalaphong and Jutapaiboon (2008), the optimum temperature for drying was 60°C. After that, the film was neutralized by 1 M of Sodium Hydroxide (NaOH) solution for 2 hours and washed with distilled water. Finally, the wet film were wiped with filter paper

and allowed to dry at room temperature for 24 hours. After the film was completely dried, the film then been peeled off from the mold.

3.3.4 FILM CHARACTERIZATION

3.3.4.1 Phenolic Content Analysis

In order to quantify the amount of total phenolic compounds contained in the bio composite produced, the Folin-Ciocalteu method was used (Wangcharoen and Morasu, 2007). 25 mg of bio composite samples was dissolved in 3 mL of distilled water at 80°C for 20 hours. 0.1 mL of the bio composite extract and 5 mL of Folin-Ciocalteu reagent was added to 7 mL of distilled water. The solution was leaved at room temperature for 8 minutes. After that, 7.5% w/v of sodium carbonate was added to the mixture. The mixture was stored for 2 hours in the dark chamber at room temperature. According to Alothman et al. (2009), the absorbance of the mixture was measured by using a spectrometer at 765 nm with the gallic acid solution (20% w/w) to construct a standard calibration curve.

3.3.4.2 Antibacterial test

The nutrient agar plate was prepared by pouring 20 mL of nutrient agar into petri dish and let the nutrient agar plate to dry at 37°C for 30 minutes. The piece of bio composite sample about 100 mm^2 was placed in the middle of the petri dish. The culture of bacteria was spread over the surface of the agar plate by using a sterile glass spreader. According to Yang and Chou (1997), the bacterium that suitable to be used in this testing is *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). The agar plates were leaved in the incubator for 24 hours at 37°C. The diameters of the inhibition zones were measured and analyzed to calculate the inhibition zone area.

3.3.4.3 Biodegradable by using *Aspergillus niger*

The ability of fungal strains to attack a bio composite material obtained was investigated by using *Aspergillus niger* as the fungal strains. The *Aspergillus niger* could change not only the bio composite surface from smoother to be rougher, but also to disrupt the bio composite (Guzun et al., 2011). *Aspergillus niger* was kept in the 4°C refrigerator until the experiment began. The dextrose-agar-potato medium was prepared by autoclave the medium at 121°C for 16 minutes. The sterile agar medium was poured into a disposal petri dish in a laminar flow hood. The *Aspergillus niger* was periodically subculture by dispersing them using sterile inoculating loop onto the fresh agar medium. Then, the sample (2cm x 2cm) was put in the middle of the disposal petri dish. The microbial grows fast in the incubator (30^{o}C) and leaved for 5 days. The weight of sample was determined in 10th days. The compound bond in the sample was checked by using Fourier Transform Infrared (FTIR) spectroscopy before and after the test.

3.3.4.4 Soil degradation Test

The degradation of plastic and bio plastic is defined as a detrimental change in its appearance, mechanical, physical properties and chemical structure. The soil used in this test was collected around Universiti Malaysia Pahang campus and put in the soil bag. The film samples in the size 2cm x 2cm were buried 5cm beneath the soil's surface and placed in the uncovered places. The test was conducted every 10 days up to 30 days of soil burial. At the regular time interval (10 days), the specimen carefully taken from the soil, washed with distilled water and dried in an oven at 60°C for 4 hours before weighed. The compound bond in the sample was checked by using Fourier Transform Infrared (FTIR) spectroscopy before and after the test.

3.3.4.5 Water Absorption Test

Water absorption measurements were performed where the dried film was immersed completely in deionized water for absorbing process at room temperature.

According to Wan et al. (2009), the samples with dimensions of 30 mm by 10 mm were cut from bio composite films. Prior to the absorption experiments, all specimens were thoroughly washed and then dried until a constant weight was attained. The test was performed for 2 hours. Every 10 minutes, the sample was removed from the water and excess water at the sample's surface was wiped out. The water uptake at any time point as a result of water absorption was determined by equation 3.1.

$$\text{Water absorption} = \frac{W_h - W_o}{W_o} \times 100 \tag{3.1}$$

Where W_h and W_0 denote, respectively as the weight of humid specimens and the original dry value (the initial weight of materials prior to exposure to moisture absorption).

Theoretically, moisture absorption processes in bio composites can be described by Fick's second law of diffusion, which is given by equation 3.2.

$$\frac{M_t}{M_m} = 1 - \frac{8}{\pi^2} \sum \frac{1}{(2n+1)^2} \exp\left\{ \frac{-D(2n+1)^2 \pi^2 t}{h^2} \right\} \tag{3.2}$$

Where M_t and M_m were the moisture content at time *t* and at equilibrium, respectively. *D* was the diffusion coefficient and *h* is the sample thickness. At short times, equation (3.2) can be reduced to equation 3.3.

$$\frac{M_t}{M_m} = \frac{4}{\pi^{1/2}} \left(\frac{Dt}{h^2} \right)^{1/2} \tag{3.3}$$

D can be calculated from the linear portion of the absorption curve (slope of M_t/M_m versus $t^{1/2}$) (Wan et al., 2009).

3.3.4.6 Quantitative of tannin

Tannins are water-soluble polyphenol that are commonly found in higher herbaceous and woody plants. Tannins have shown potential antiviral, antibacterial and ant parasitic effects. The procedures to determine the tannin compound in Areca nut extract was shown in the research conducted by Wetwitayaklung et al. (2006). Crude seed extracts were dissolved in distilled water and filtrated to get clear solution. The 10-20 mL of the solution (equal to tannins 0.01 g) was added to 25 mL indigo carmine solution. The indigo carmine solution was prepared by dissolved 1 g of indigo carmine in 1 L of H_2SO_4 acidic solution (0.05 M). Then the solution was diluted with 500-700 mL water to get clear solution. This solution was titrated with 0.04 M $KMnO_4$ solution until the solution became pale yellow (end point). The used volume in ml of $KMnO_4$ solution (A) was recorded. The 60 mL of clear solution was added to 25 mL gelatin solution. Then, filter aid was added, and the mixture was shook for 15 min and filtrated. The filtrate of 50 mL was added to 20 mL indigo carmine solution and then diluted this solution with 500-700 mL water. The solution was titrated with 0.04 M $KMnO_4$ solution until the solution became pale yellow (end point). The used volume of $KMnO_4$ solution (B) was recorded. The amount of tannins that was oxidized with $KMnO_4$ was calculated from the equation 3.4.

$$\text{Concentration of tannin} = \frac{(A\text{-}B) \times (\text{g tannin/ml } KMnO_4)}{\text{ml of sample solution}} \qquad (3.4)$$

Where A= total tannin like materials, B= non tannin materials, A-B= true tannins

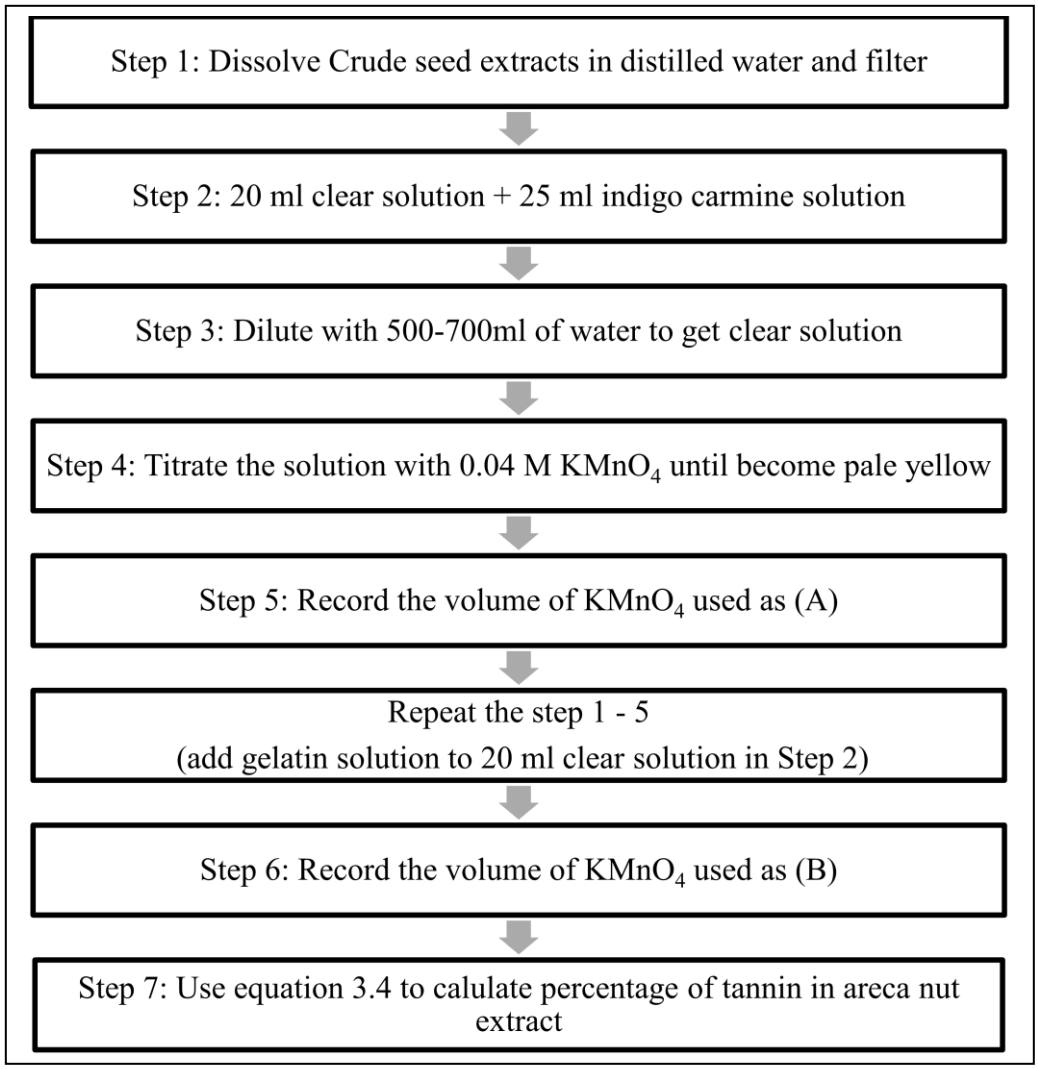

Figure 3.2: Experimental procedure for quantitative of tannin test

3.3.4.7 Fourier Transform Infrared Spectroscopy (FTIR) analysis

Fourier Transform Infrared Spectroscopy (FTIR) was performed using FTIR Thermo Electron Corporation. FTIR spectra were recorded between 700 and 3600 cm^{-1} with a piece of film 2 cm x 2cm in size.

3.3.4.8 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was performed using SEM EDX Spectrometer EVO 50, operating at an acceleration voltage of 15kV. SEM was used to characterize the microstructure of the samples. According to Phisalapong and Jatupaiboon (2008), the film sample was frozen in liquid nitrogen. Immediately, the sample was snapped, vacuum dried and then sputter with gold. The surface and cross section of biocomposite samples were observed with 100x and 500x resolution.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter provides a detail discussion about the results testing and analysis of antibacterial bio composite from bacterial cellulose (BC) and *Areca Catechu* extract by using phenolic content test, testing for antibacterial activity, biodegradation by using fungus, soil degradable test, water absorption test, quantitative of tannin test, Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM). The results obtained were compared with the findings from previous researchers.

4.2 PHENOLIC CONTENT TEST

The concentrations of total phenolic content are summarized in Table 4.1 and Figure 4.1.

Table 4.1: The phenolic concentration for each sample

Bacterial cellulose (BC)	Areca nut extract	Optical Density (OD)	Concentration (mg/L)
0 g	0%	0	0
	25%	1.288	52.1
	50%	1.505	122.2
	75%	1.756	203.2
	100%	2.101	314.5
7 g	0%	0	0
	25%	1.330	65.7
	50%	1.548	135.9
	75%	1.800	217.3
	100%	2.124	321.9
14 g	0%	0	0
	25%	1.363	76.2
	50%	1.587	148.5
	75%	1.844	231.6
	100%	2.206	348.4
21 g	0%	0	0
	25%	1.414	92.9
	50%	1.628	161.9
	75%	1.882	243.9
	100%	2.251	362.9
28 g	0%	0	0
	25%	1.441	101.6
	50%	1.663	173.2
	75%	1.950	265.8
	100%	2.309	381.5

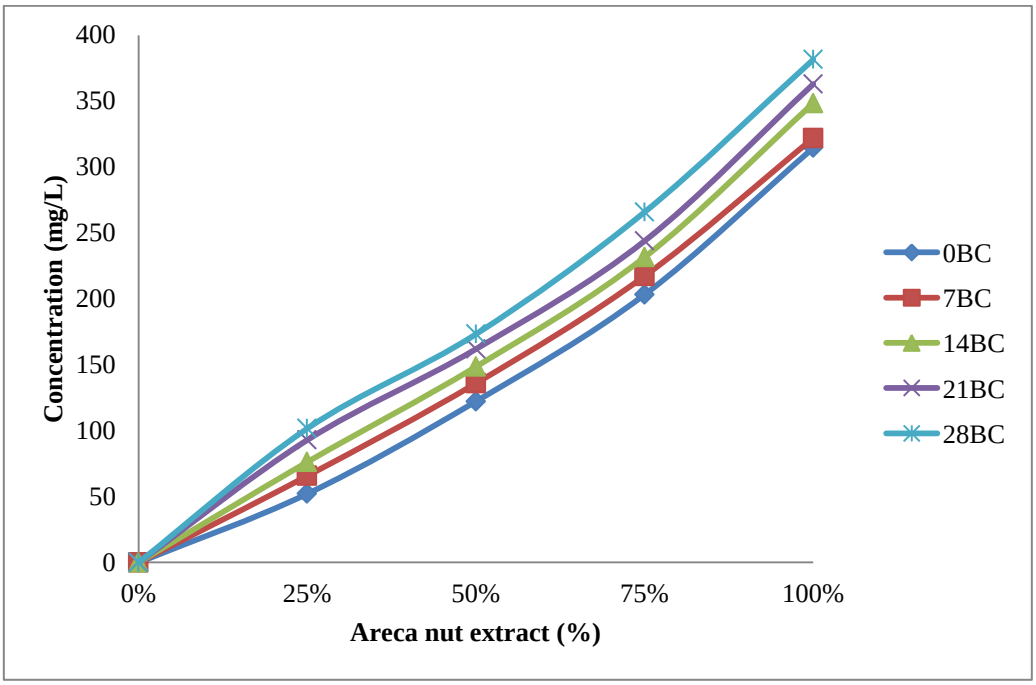

Figure 4.1: The phenolic concentration for each sample

From Table 4.1, the highest concentration of phenolic content was found in the sample with composition of 28 grams bacterial cellulose (BC) and 100% areca nut extract which was 381.3 mg/L. Based on the Figure 4.1, the concentration of phenolic content was increased when the percentage of areca nut extract increased. According to Jaiswal et al. (2011), the polyphenols of areca catechu nut are mainly flavonoids and their concentration decreases with the maturity of the nuts. The extract was extracted from 6 month areca nuts. When the composition of bacterial cellulose (BC) in the sample was increased, the total phenolic content also increased. Based on research conducted by Elegir et al. (2008), the phenolic structures have a potential to be grafted on the lignocellulosic fibre surface to develop covalently bound antimicrobial fibre based products. The number of covalent bond between cellulose fibre and phenolic compound was the highest in the sample with 28 grams bacterial cellulose (BC) and 100% areca nut extract due to the presence of high percentage of cellulose structure from bacterial cellulose (BC) and phenolic compound from areca nut extract.

4.3 ANTIBACTERIAL TEST

The antibacterial properties of the samples prepared were analyzed by using disc diffusion method. The antibacterial activities of bio composite were evaluated by using two different bacteria; *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). The inhibition on the agar plate was investigated for 5 days to see any potential antibacterial effects of the bio composite against the representative for both gram positive and gram negative bacteria.

Table 4.2: Zone of inhibition area for *S. aureus*

Bacterial cellulose (BC)	Areca nut extract	Zone of Inhibition (cm^2)		
		Day 1	Day 3	Day 5
0 g	0%	0	0	0
	25%	0	0	0.3
	50%	0	0.2	0.7
	75%	0	0.4	1.1
	100%	0	0.7	1.5
7 g	0%	0	0	0
	25%	0	0	0.3
	50%	0	0.3	0.9
	75%	0	0.4	1.3
	100%	0	0.8	1.8
14 g	0%	0	0	0
	25%	0	0	0.4
	50%	0	0.4	1.0
	75%	0	0.7	1.6
	100%	0	1.0	2.3
21 g	0%	0	0	0
	25%	0	0.1	0.6
	50%	0	0.5	1.0
	75%	0	0.9	1.7
	100%	0	1.2	2.5
28 g	0%	0	0	0
	25%	0	0.2	0.7
	50%	0	0.5	1.2
	75%	0	1.0	1.8
	100%	0	1.6	2.9

Table 4.3: Zone of inhibition area for *E. coli*

Bacterial cellulose (BC)	Areca nut extract	Zone of Inhibition (cm^2)		
		Day 1	Day 3	Day 5
0 g	0%	0	0	0
	25%	0	0	0
	50%	0	0	0.3
	75%	0	0.4	0.9
	100%	0	0.5	1.2
7 g	0%	0	0	0
	25%	0	0	0.1
	50%	0	0.2	0.5
	75%	0	0.4	0.9
	100%	0	0.6	1.3
14 g	0%	0	0	0
	25%	0	0	0.2
	50%	0	0.3	0.7
	75%	0	0.6	1.1
	100%	0	0.8	1.6
21 g	0%	0	0	0
	25%	0	0	0.3
	50%	0	0.3	0.8
	75%	0	0.8	1.4
	100%	0	1.0	1.7
28 g	0%	0	0	0
	25%	0	0	0.3
	50%	0	0.4	0.9
	75%	0	1.1	1.5
	100%	0	1.2	2.2

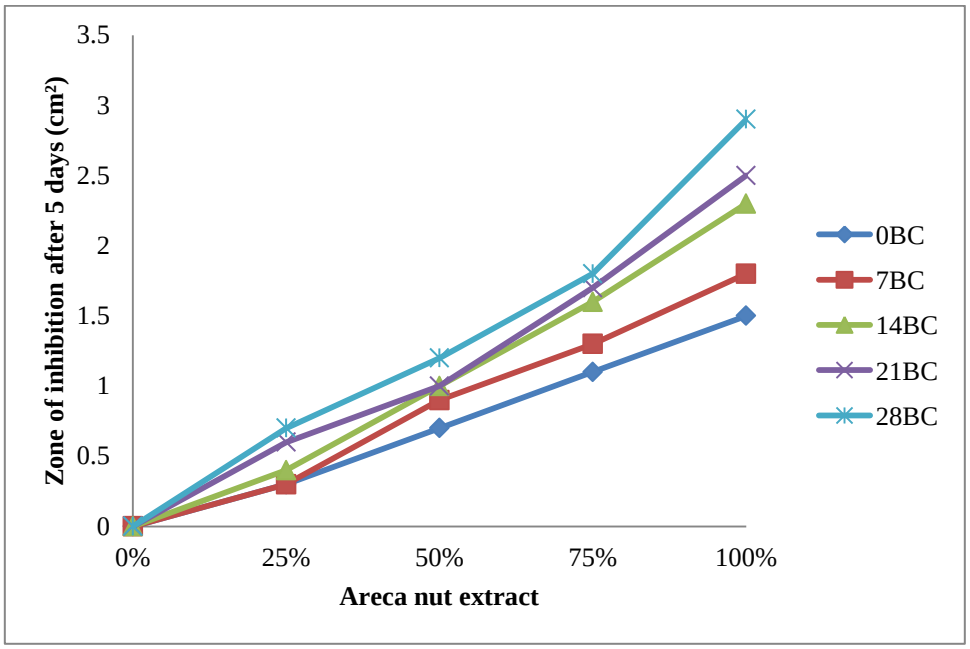

Figure 4.2: Zone of inhibition of *S. aureus* after 5 days

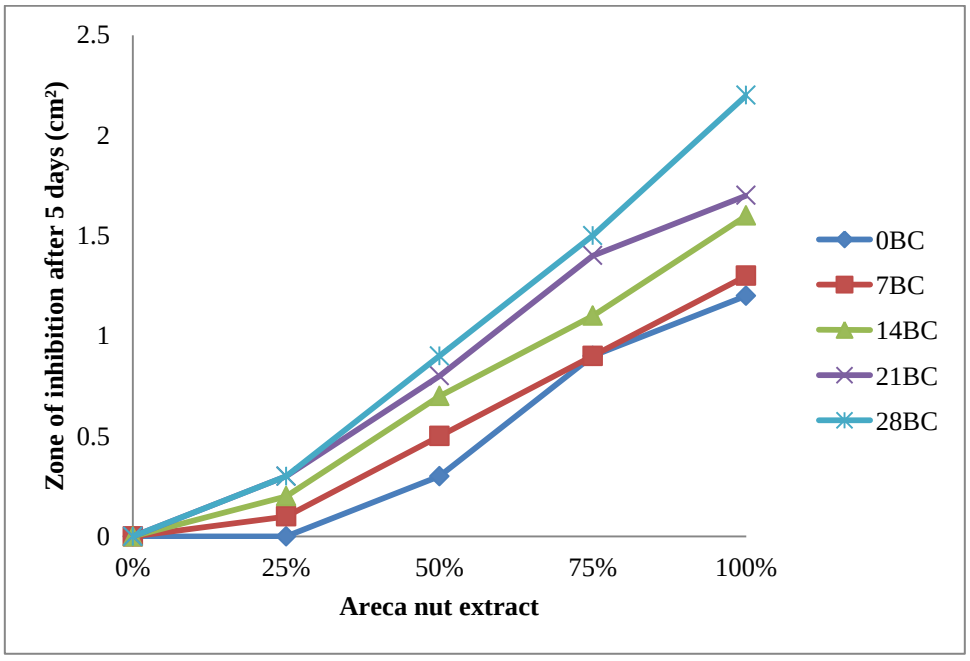

Figure 4.3: Zone of inhibition of *E. coli* after 5 days

All the antibacterial results obtained from the disc diffusion method were summarized in Table 4.2 and Table 4.3. From the Figure 4.2 and Figure 4.3, the zones of inhibition area were increased directly proportional with the increasing of areca nut extract. For gram positive bacteria *S. aureus*, the maximum zone of inhibition areas were observed with bio composite containing 28 grams bacterial cellulose (BC) and 100% areca nut extract that was more than 2.9 cm^2 while for gram negative bacteria *E. coli*, the maximum inhibition area was 2.2 cm^2 that observed with the same bio composite composition. Based on Table 4.2 and Table 4.3, the areas of inhibition after 1 day cultivation were visible for both of the gram positive and gram negative bacteria. There were no zones of inhibition area for bio composite containing 0% of areca nut extract on the agar surface due to the absence of phenolic content as the antibacterial properties in areca nut extract (Papadopoulou et al., 2005).

Figure 4.2 and Figure 4.3 illustrated that the gram negative bacteria *E. coli* showed the lower zones of inhibition area than gram positive bacteria *S. aureus* due to the presence of areca nut extract. It showed that the bio composite obviously more effective against gram positive bacteria *S. aureus* than gram negative bacteria *E. coli*. This observation can be attributed to differences in the structure of bacteria cell wall. The cell walls of gram-positive bacteria are made up of twenty times as much murein or peptidoglycan than gram-negative bacteria. The less complex structure of the cell wall in the gram-positive bacteria makes it more permeable to the antimicrobial compounds (Papadopoulou et al., 2005).

4.4 BIODEGRADABLE BY USING *ASPERGILLUS NIGER*

Aspergillus niger were used to investigate the ability of fungal strains to attack a bio composite material obtained from bacterial cellulose (BC) and areca nut extract. The biodegradable test was performed for 10 days.

Table 4.4: Weight loss of bio composite by using *Aspergillus niger*

Areca nut extract	Bacterial cellulose (BC)	Initial weight (g)	Final weight (g)	Percentage weight loss (%)
0%	0 g	0.1190	0.0556	53.3
	7 g	0.1500	0.0745	50.3
	14 g	0.1450	0.0812	44.0
	21 g	0.1580	0.0959	39.3
	28 g	0.1290	0.0935	27.5
25%	0 g	0.1190	0.0589	50.5
	7 g	0.1410	0.0749	46.9
	14 g	0.1700	0.0997	41.4
	21 g	0.0970	0.0619	36.2
	28 g	0.0980	0.0749	23.6
50%	0 g	0.1040	0.0591	43.2
	7 g	0.1240	0.0721	41.9
	14 g	0.1230	0.0775	37.0
	21 g	0.1350	0.0916	32.1
	28 g	0.1720	0.1353	21.3
75%	0 g	0.1070	0.0659	38.4
	7 g	0.1560	0.0998	36.0
	14 g	0.1070	0.0708	33.8
	21 g	0.1140	0.0829	27.3
	28 g	0.1330	0.1081	18.7
100%	0 g	0.1180	0.0791	33.0
	7 g	0.0980	0.0684	30.2
	14 g	0.1110	0.0808	27.2
	21 g	0.1450	0.1095	24.5
	28 g	0.1080	0.0894	17.2

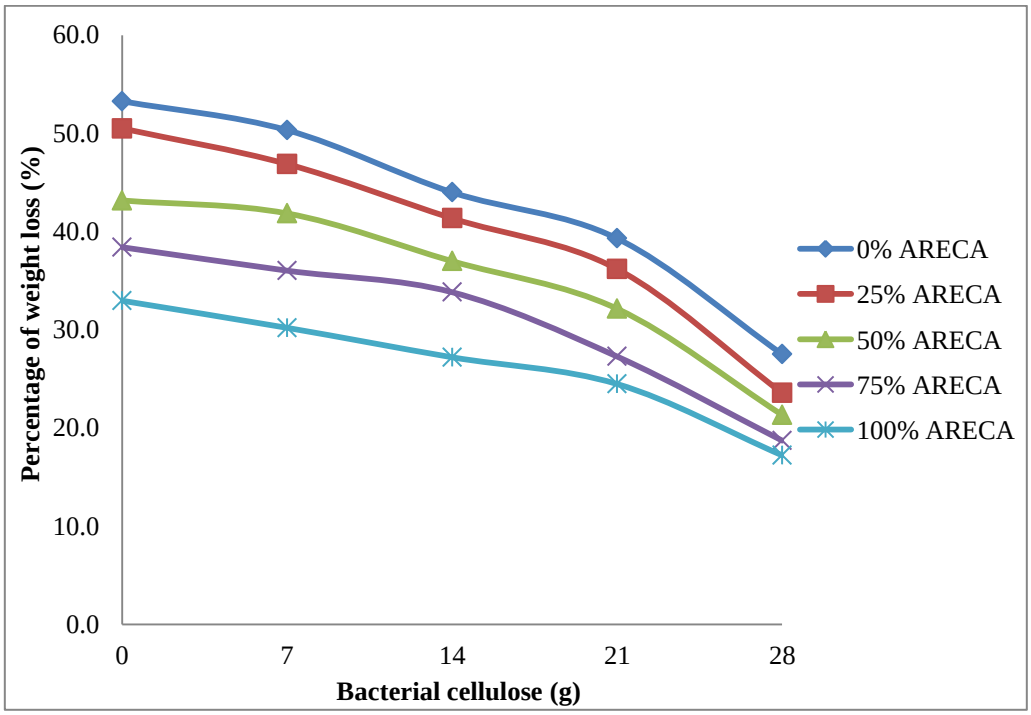

Figure 4.4: The percentage weight loss for each sample

Table 4.4 shows the weight loss of the bio composites after biodegradation test by using *Aspergillus niger*. From the Figure 4.4, the highest composition of bacterial cellulose (BC) and areca nut extract shows the lowest weight loss after degradation with *Aspergillus niger* for 10 days. The samples that contain smallest proportion of bacterial cellulose (BC) and areca nut extract degraded faster than the other samples. According to Guzun et al. (2011), the degree of microbial degradation is dependent on the culture medium and on composition of composite materials. Thus, the process was facilitated by the presence of bio cellulose in the film composition with an easily available carbon sources. Based on research conducted by Vries & Visser, (2001), there are four classes of enzymes are involved in biodegradation of cellulose. Endoglucanases hydrolyze cellulose to glucooligosaccharides, cellobiohydrolases release cellobiose from crystalline cellulose, glucosidases degrade the oligosaccharides to glucose and exoglucanases release glucose from cellulose and glucooligosaccharides

Regarding the different behavior of bio composite in connection with bacterial cellulose/ areca nut extract proportion, the composite that contains the highest proportion of areca nut extract was more resistant to fungal attack which showed the lowest degradation. According to Wetwitayaklung et al. (2006), the areca nut extract has anthelmintic, antifungal, antibacterial, anti-inflammatory and antioxidant activities. Based on these experimental results it can be concluded that *Aspergillus niger* can be used for biodegradation of bacterial cellulose (BC)/ areca nut extract and starch based composites. This was proved by Gu (2003) mentioned that microorganisms such as bacteria and fungi are involved in the degradation of both natural and synthetic plastics.

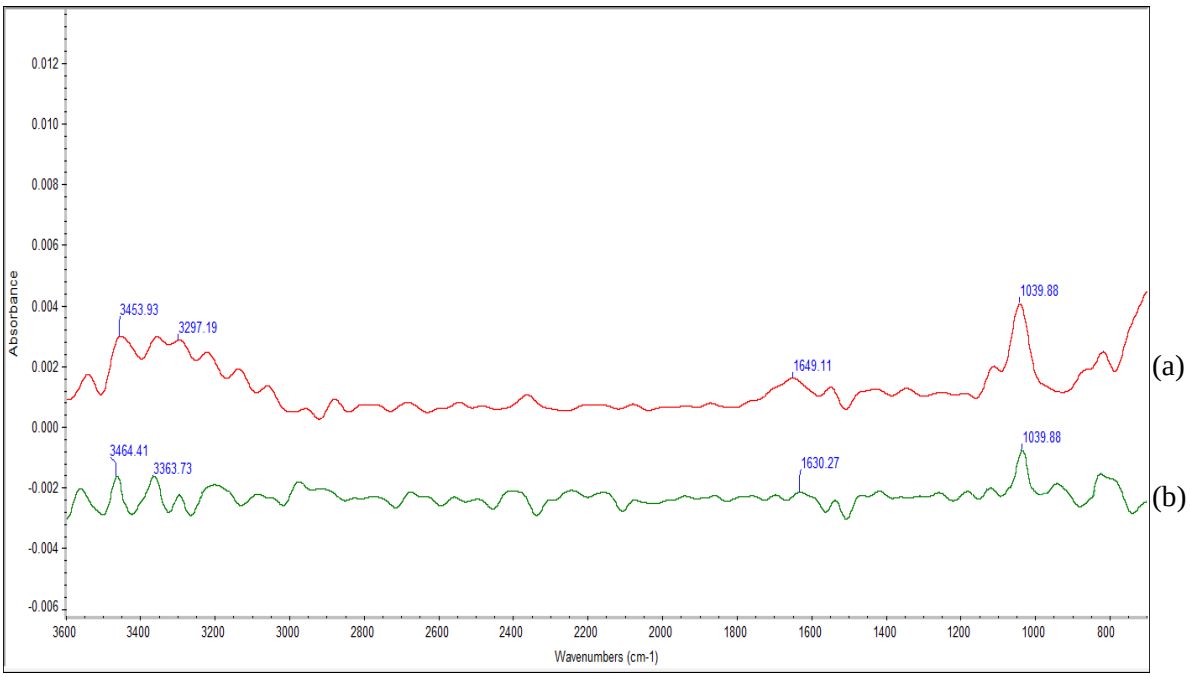

Figure 4.5: FTIR spectra of bio composite sample degradation after 10 days by using *Aspergillus niger* (a) before degradation, (b) after degradation

The degradation of bio composite sample, molecular interaction and crystallinity of bacterial cellulose (BC) and areca nut extract bio composites were investigated using Fourier transform infrared spectroscopy in order to determine the effect of bacterial attack during degradation process. In Figure 4.5 (a), there were O-H, C-H and C=O stretching

mode which characterized as the major steps of the degradation process. There were changed in peak heights in the spectra before and after fungal attack. All the samples showed a reduction in the hydroxyl (3100-3500 cm^{-1}) and stretching of C=O (1082 cm^{-1}) due to the degradation of the sample (Guzun et al., 2011). Mohan and Srivastava (2010) stated that during degradation, enzymes from microorganisms break down complex polymers yielding smaller molecules of short chain and then to be utilized as carbon and energy sources for the fungus.

4.5 SOIL BURIAL DEGRADABLE TEST

The soil burial degradable test was performed around the university area for 30 days. The biodegradability of the bio composite samples was determined by visual changes and weight loss of due to the burial degradable in soil.

Table 4.5: Initial weight, final weight and percentage of weight loss of bio composite

Areca nut extract	Bacterial cellulose (BC)	Initial Weight (g)	Final Weight (g)	Weight Loss (%)
0%	0 g	0.1802	0.0918	49.1
	7 g	0.1764	0.1017	42.3
	14 g	0.2763	0.1689	38.9
	21 g	0.2407	0.1501	37.6
	28 g	0.1926	0.1254	34.9
25%	0 g	0.2057	0.1152	44.0
	7 g	0.2667	0.1637	38.6
	14 g	0.2619	0.1641	37.3
	21 g	0.1946	0.1289	33.8
	28 g	0.1893	0.1337	29.4
50%	0 g	0.2191	0.1314	40.0
	7 g	0.2072	0.1344	35.1
	14 g	0.1883	0.1276	32.2
	21 g	0.1922	0.1353	29.6
	28 g	0.1988	0.1453	26.9
75%	0 g	0.1914	0.1227	35.9
	7 g	0.2264	0.1555	31.3
	14 g	0.1446	0.1052	27.2
	21 g	0.1544	0.1181	23.5
	28 g	0.1887	0.1482	21.5
100%	0 g	0.1438	0.0995	30.8
	7 g	0.1576	0.1175	25.4
	14 g	0.223	0.1701	23.7
	21 g	0.1705	0.1375	19.4
	28 g	0.2122	0.1742	17.9

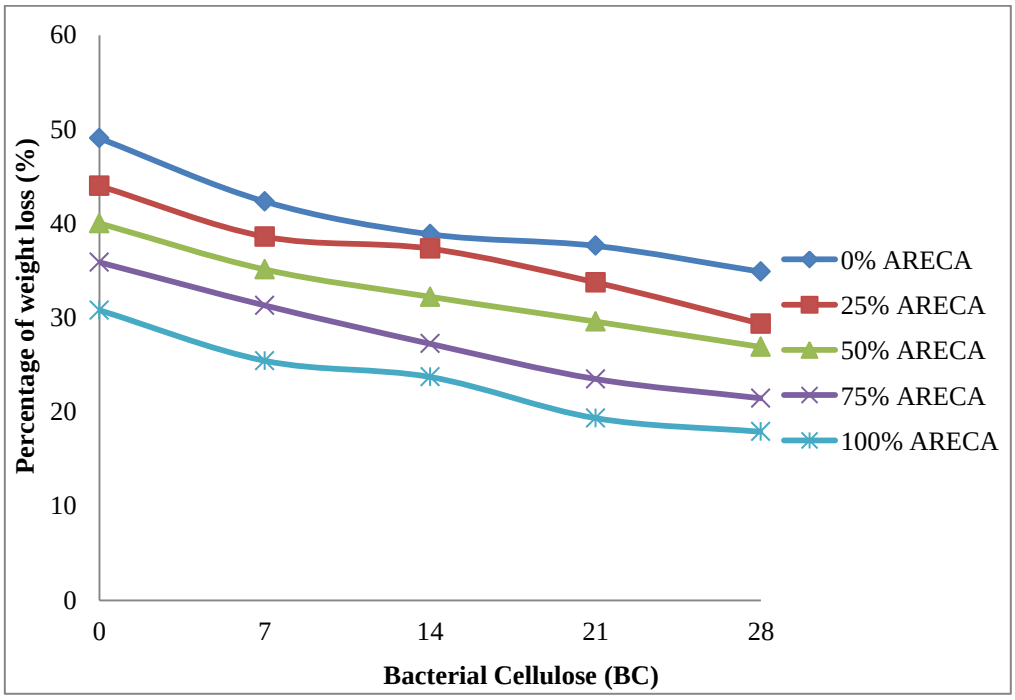

Figure 4.6: Percentage weight loss for each sample

The soil burial degradation tests were performed for the composite containing different composition of bacterial cellulose (BC) and areca nut extract. The bio composite undergoes degradation in the soil from the action of microorganisms such as bacteria, fungi, and algae. The weight loss of the bio composite samples after the soil burial degradable test for 30 days was showed in Table 4.5. From the Figure 4.6, the weight loss of bio composite samples shows a linear relation with degradation time for bacterial cellulose (BC) and areca nut extract bio composites. The bio composite samples with higher percentages of areca nut extract also show the low weight loss than the bio composite samples contain lower percentages of areca nut extract. According to Tunç and Duman (2011), there are four potential type chemical interactions between phenolic compound in areca nut extract and bacterial cellulose (BC) which is covalent, ionic, hydrogen bonding and hydrophobic interaction. The reaction between cellulose side and phenolic compound build the hydrogen bonds between carbonyl group of polypeptide chain and phenolic hydroxyl group.

In the other hand, the bio composite sample that contains high proportional of bacterial cellulose (BC) degrades slower than the low proportional of bacterial cellulose (BC). This caused by the crystalline regions in the bacterial cellulose (BC) molecules (Wan et al., 2009). Bacterial cellulose (BC) molecules have two regions: one of which, called 'crystalline cellulose' is composed of highly-oriented molecules, and the other one, called 'amorphous cellulose', comprises less-oriented molecules. According to Alvarez et al, (2006), the crystalline regions are more difficult to degrade due to the higher resistance to microorganism attacks.

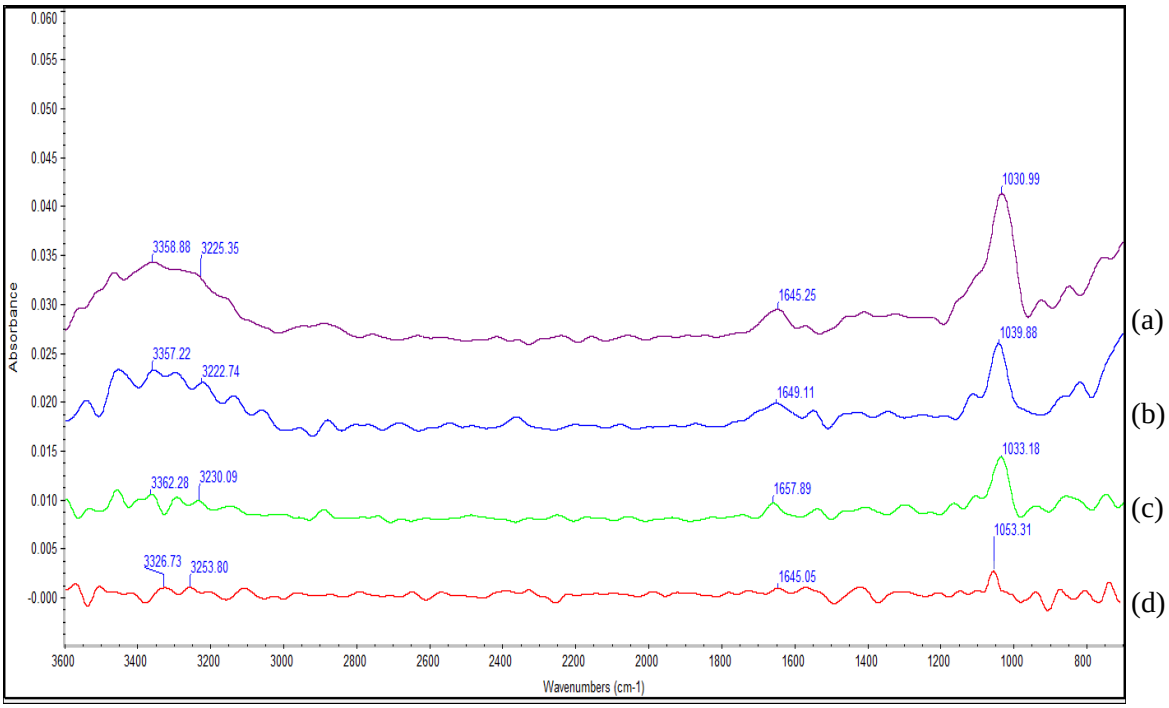

Figure 4.7: FTIR spectra of bio composite sample during soil burial degradation test (a) before test, (b) after 10 days, (c) after 20 days, (d) after 30 days

In order to determine the effect of soil microorganism attack on the bacterial cellulose (BC) and areca nut extract bio composites during degradation process, Fourier Transform Infrared spectroscopy was used. In Figure 4.7, there were changed in peak heights in the spectra before and after fungal attack. All the samples showed a reduction in the hydroxyl (3100-3500 cm^{-1}), carbonyl (1642 cm^{-1}) and stretching of C=O (1082 cm^{-1})

due to the degradation from the soil microorganism. According to Alvarez et al. (2006), bacterial cellulose (BC) degraded more slowly than starch molecules due to the crystalline regions in bacterial cellulose (BC) molecules. From the figure, the peak heights of C=O and hydroxyl group reduced faster than carbonyl group. The loss of carbonyl group was caused by the decomposition of carbohydrates due to presence of amylase enzyme. According to Aiyer (2005), amylase degrades starch and related polymers to yield products characteristic of individual amylolytic enzymes.

4.6 WATER ABSORPTION TEST

Water absorption measurements were performed where the dried film was immersed completely in deionized water for absorbing process at room temperature. The test was performed for at least 2 hours to make sure that the water absorption uptake was maximum. Theoretically, water absorption processes in bio composites can be described by Fick's second law of diffusion (Wan et al., 2009).

Table 4.6: Water absorption and diffusivity of each sample

Areca nut extract	Bacterial cellulose (BC)	Water absorption (%)	Diffusivity, D x 10^6 (mm^2/min)
0%	0	70.7	4.52
	7	63.5	4.47
	14	58.6	4.34
	21	49.9	4.27
	28	49.9	4.19
25%	0	65.8	4.58
	7	59.3	4.50
	14	56.2	4.44
	21	47.4	4.22
	28	47.3	4.19
50%	0	59.7	4.71
	7	51.1	4.60
	14	48.3	4.40
	21	46.3	4.41
	28	44.4	4.41
75%	0	58.1	4.76
	7	50.0	4.57
	14	47.1	4.52
	21	42.4	4.46
	28	42.5	4.42
100%	0	52.9	4.96
	7	47.8	4.76
	14	40.9	4.72
	21	35.7	4.69
	28	35.1	4.63

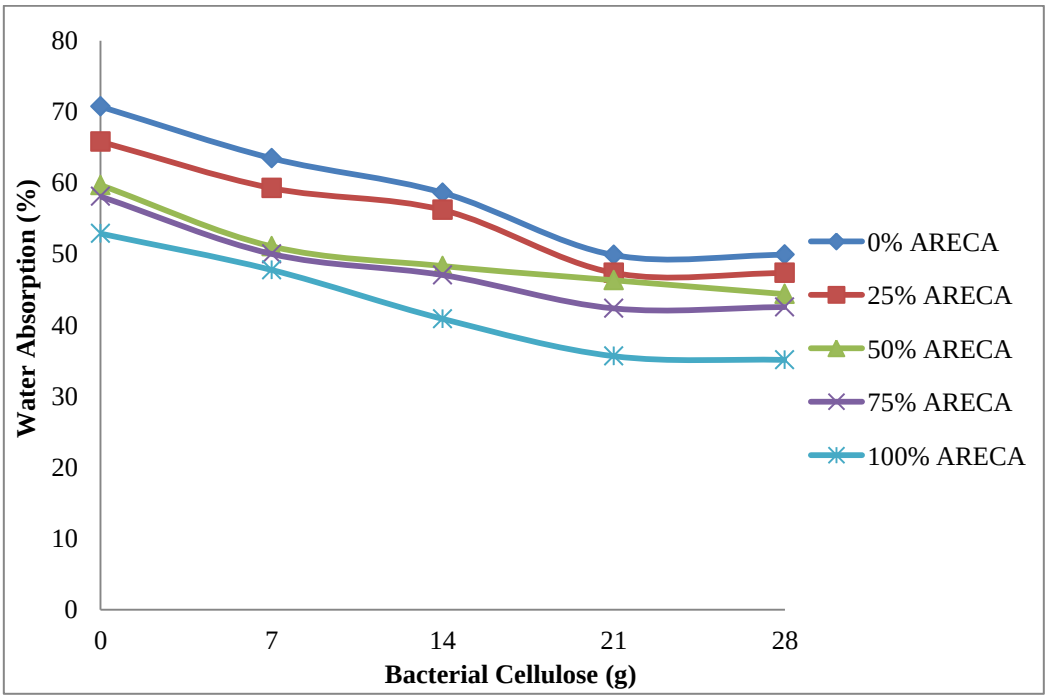

Figure 4.8: Percentage of water absorption after 2 hours

Table 4.6 shows the percentage of water absorption and diffusivity (D) of each bio composite samples. Both of percentage of water absorption and diffusivity (D) were decreased when the composition of bacterial cellulose (BC) in the bio composite samples increased. The percentage of water absorption dropped from 70.7% to 49.9% when the composition of bacterial cellulose (BC) was increased from 0 gram to 28 grams. The value of diffusivity (D) was highest at bio composite sample with 0 gram of bacterial cellulose (BC) and 0% of areca nut extract. This is because of the hydrophilic properties of starch thus enhancing the higher absorption of water molecules by starch (Euaphantasate et al., 2008). According to Wan et al. (2009), both starch and bacterial cellulose (BC) are hydrophilic which may render the bio composites with high moisture absorption properties at higher proportional of bacterial cellulose (BC). However, the chemistry similarity may result in good interface adhesion between the two components, which can prevent moisture absorbance thus lower the diffusivity (D) of the bio composite samples. This can be proved by the value of diffusivity (D) from Table 4.6. The value of diffusivity (D) was decreased when the composition of bacterial cellulose (BC) increased.

From the results, it was observed that the bio composite samples that contain higher percentage of areca nut extract showed the lower percentage of water absorption. The highest percentage of water absorption for 14 grams bacterial cellulose (BC) with 0% areca nut extract and 100% areca nut extract are 58.6% and 40.9%, respectively. The same goes with the value of diffusivity (D). The bio composite sample with composition of 14 grams bacterial cellulose (BC) and 0% of areca nut extract has diffusivity value about 3.12×10^{-6} cm^2/min while the bio composite sample with composition of 14 grams of bacterial cellulose (BC) and 100% of areca nut extract has diffusivity value of 3.84×10^{-7} cm^2/min. The presence of strong hydrogen bond from cellulose carbonyl group and phenolic hydroxyl group was related to the decreasing of percentage of water absorption and diffusivity (D). Based on researches conducted by Gardner et al. (2008), cellulose has a strong affinity to itself and hydroxyl containing materials to form a strong hydrogen bond. The strong hydrogen bonds prevent the water molecules to bind with starch thus decreasing the percentage of water absorption and diffusivity (D).

4.7 QUANTITATIVE OF TANNIN

The concentration of tannin for bio composite produce from bacterial cellulose (BC) and areca nut extract were summarized in Table 4.7 and Figure 4.9.

Table 4.7: The tannin concentration of each bio composite sample

Bacterial cellulose (BC)	Areca nut extract (%)	Concentration (mg/L)
0 g	0%	0
	25%	59.8
	50%	136.8
	75%	219
	100%	343.9
7 g	0%	0
	25%	73.7
	50%	147.4
	75%	238.7
	100%	362.5
14 g	0%	0
	25%	84.1
	50%	161.9
	75%	253.2
	100%	388.1
21 g	0%	0
	25%	98.1
	50%	175.2
	75%	271.1
	100%	402.3
28 g	0%	0
	25%	111.3
	50%	188.9
	75%	297.3
	100%	423.9

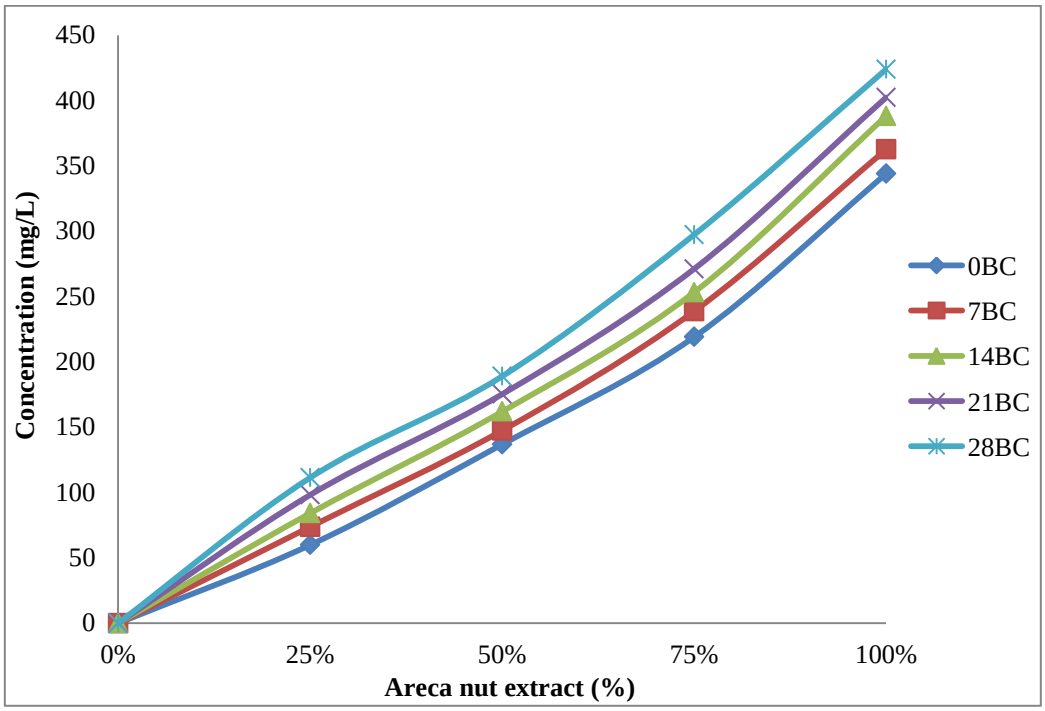

Figure 4.9: Tannin concentration for each bio composite samples

From Table 4.9, the highest concentration of tannin content was found in the sample with composition of 28 grams bacterial cellulose (BC) and 100% areca nut extract which was 423.9 mg/L while the lowest was found in sample with 0 gram bacterial cellulose (BC) and 25% areca nut extract which was 59.8 mg/L. Based on the Figure 4.9, the concentration of tannin content was increased when the percentage of areca nut extract increased. Based on research conducted by Jaiswal et al. (2011), tannin is one of the main constituents of the areca nut. The areca nut contains about 15% of tannins. According to Wetwitayaklung et al. (2006), the ethanol extracts of 6 to 8 month areca nut contains the highest percentage of tannin. When the composition of bacterial cellulose (BC) in the sample was increased, the total phenolic content also increased. Same as polyphenol, tannin also has hydroxyl group. Hydroxyl group from tannin have ability to form a hydrogen bond with carbonyl group from cellulose (Gardner et al., 2008).

4.8 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Fourier Transform-Infrared Spectroscopy (FTIR) is an analytical technique used to analyzing the interactions between antibacterial bio composite produced by different composition of bacterial cellulose (BC) and areca nut extract.

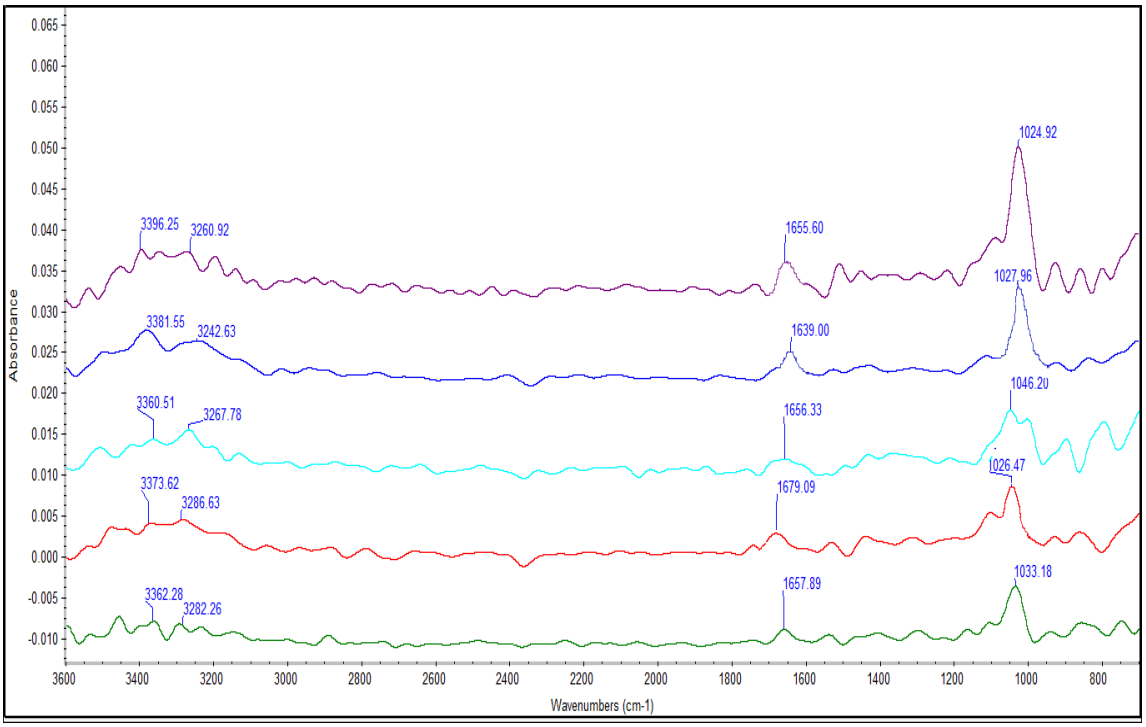

Figure 4.10: The FT-IR spectra of 14 grams of bacterial cellulose (BC) with different of areca nut extract percentages; (a) 100%, (b) 75%, (c) 50%, (d) 25% and (e) 0%

The Figure 4.10 illustrated the FTIR spectra for 14 grams bacterial cellulose with different percentages of areca nut extract. The FTIR spectra of all bio composite samples were detecting at wavenumbers ranging from 3600 to 700 cm^{-1}. For the close observation, the expansion of the FTIR spectra of the bio composite samples at wave number ranging from 1700 to 1500 cm^{-1} was displayed in Figure 4.7. According to Klemm et al. (2001), the intense absorption in the spectrum of cellulose was the band at 1642.9 cm^{-1}, which has been assigned to carbonyl group. In Figure 4.10 (a-d), the bio composite samples developed by

the supplement of areca nut extract of 25%, 50%, 75% and 100% present a peak shift and an occurrence of new peak from 3200 to 3500 cm^{-1}. The presence of two new peaks in FTIR spectra region when bio composite samples were develop by the supplement of 25 – 100% areca nut extract implied the incident of the intermolecular interaction of bacterial cellulose (BC) and the hydroxyl group of areca nut extract (Guzun et al., 2011).

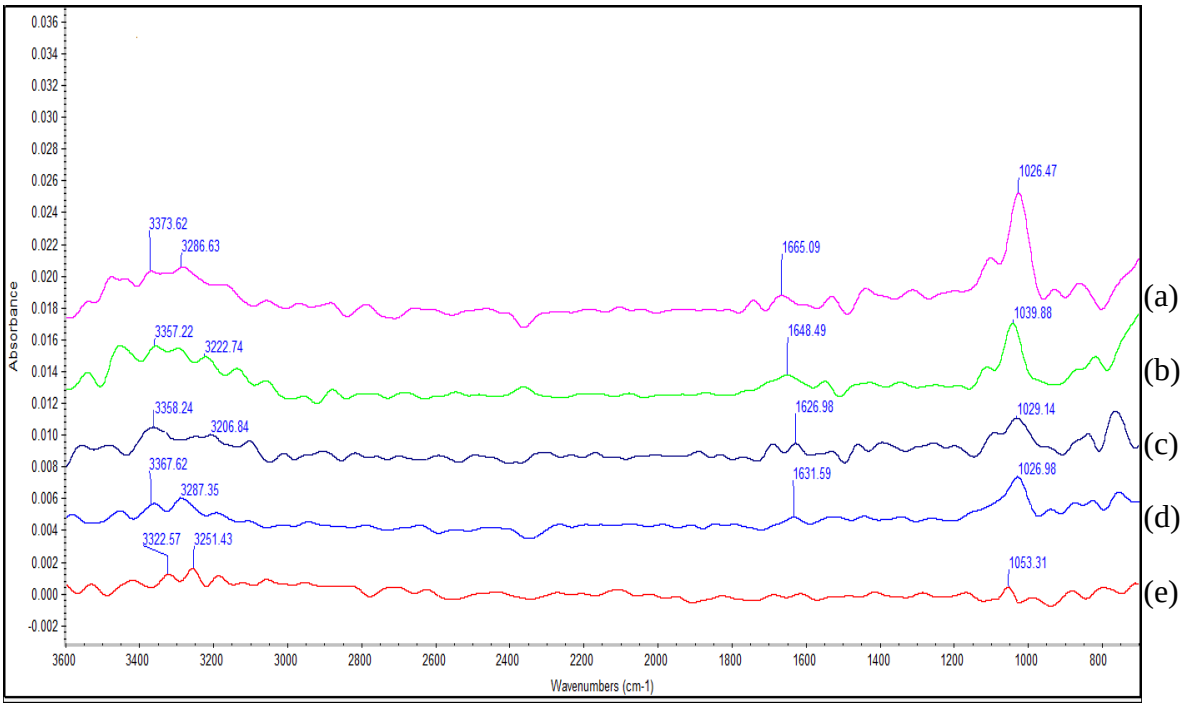

Figure 4.11: The FT-IR spectra of 100% areca nut extract with different of composition of bacterial cellulose (BC); (a) 28 g BC, (b) 21 g BC, (c) 14 g BC, (d) 7 g BC and (e) 0 g BC

Figure 4.11 shows the FTIR spectra of different composition of bacterial cellulose (BC) used in the preparation of bio composite samples in order to determine the effect of molecules interaction between bacterial cellulose (BC) and areca nut extract. In the spectra, there were identified three vibrational modes which were the O–H, C–H and C=O stretching modes. Areca nut extract has been predicted to interact extensively with bacterial cellulose (BC), as both have the ability to form hydrogen bonds, considering their OH groups (Gardner et al., 2008). Based on research conducted by Guzun et al. (2011), the

characteristic bands of bacterial cellulose are in cm^{-1}: 3,326 due to O–H stretching, 2,896 due to CH stretching and 1,102, due to C–C stretching vibration, skeletal/vibrations and ring vibrations. All the bio composite samples showed an increasing in the carbonyl group (1600 - 1800 cm^{-1}) and C-O-C symmetric stretching (1081 cm^{-1}) due to the addition of bacterial cellulose (BC) in the samples (Panaser et al., 2011). The peak at 1642 cm^{-1} could also be contributed to the water absorbed in the amorphous region of the bacterial cellulose (BC) fibrils.

4.9 SCANNING ELECTRON MICROSCOPY (SEM)

The observation of the bio composite surfaces and fracture were performed with scanning electron microscope.

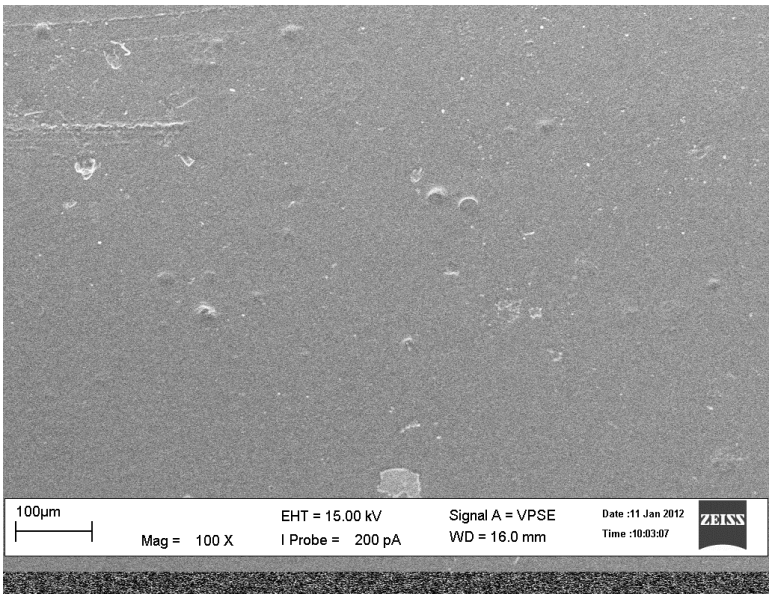

Figure 4.12: Scanning electron micrograph of surface of 0BC+100% areca nut

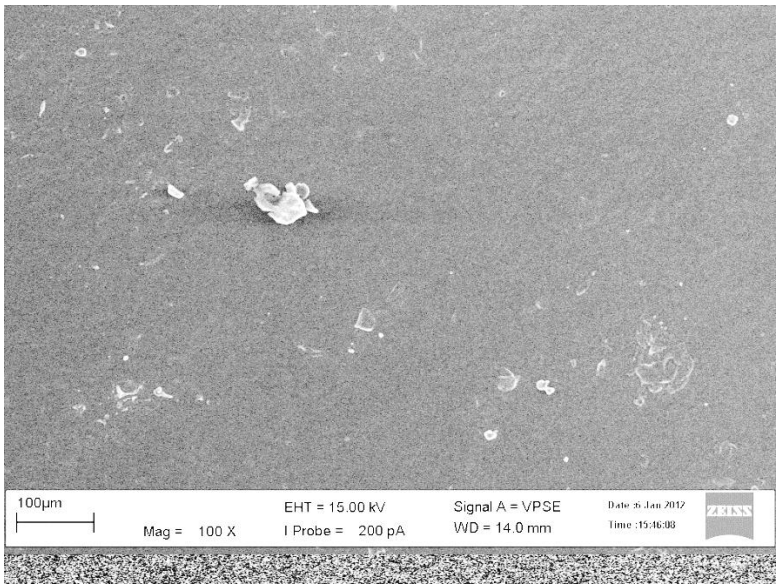

Figure 4.13: Scanning electron micrograph of surface of 14BC+100% areca nut

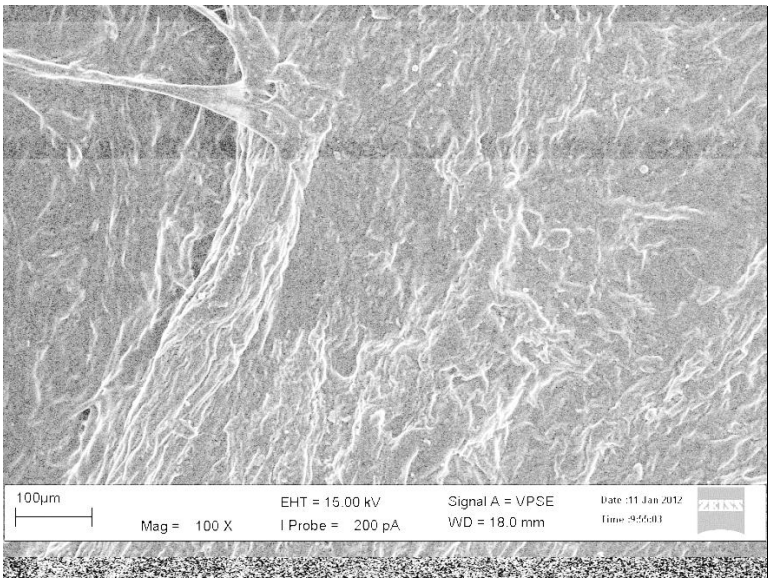

Figure 4.14: Scanning electron micrograph of surface of 28BC+100% areca nut

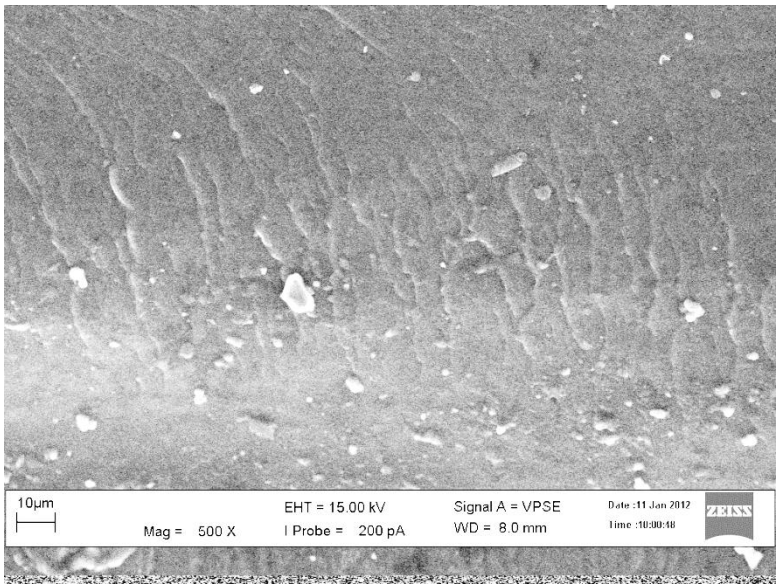

Figure 4.15: Scanning electron micrograph of cross section of 0BC+100% areca nut

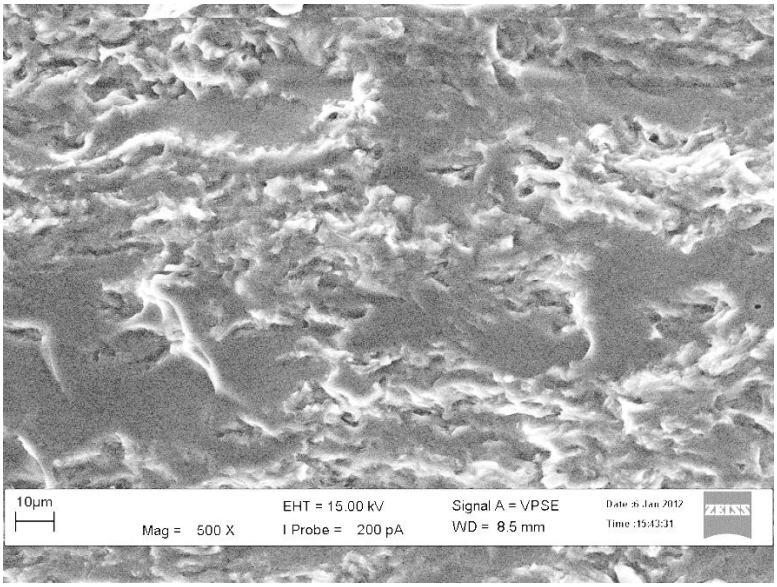

Figure 4.16: Scanning electron micrograph of cross section of 14BC+100% areca nut

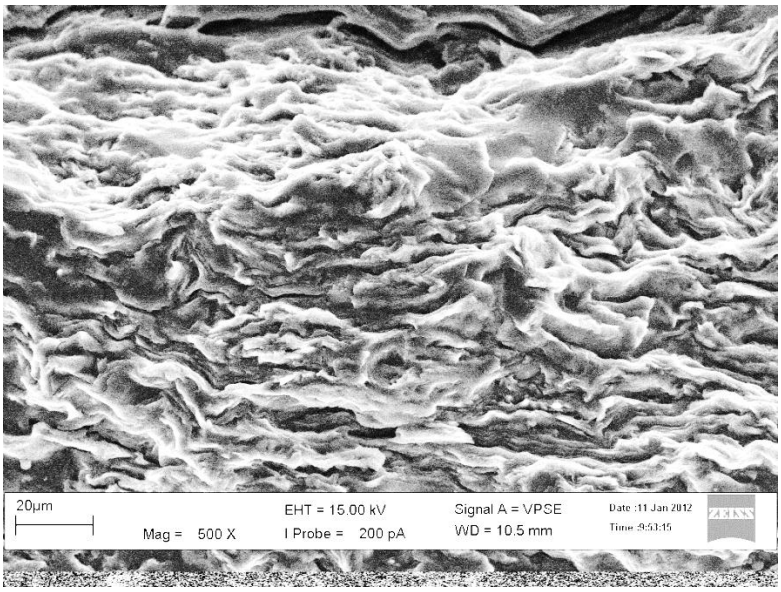

Figure 4.17: Scanning electron micrograph of cross section of 28BC+100% areca nut

SEM images in Figure 4.12 to Figure 4.17 illustrated the differences in the surface and cross section morphology of the developed bio composite samples contained 0 gram, 14 grams and 28 grams of bacterial cellulose (BC) in the samples, respectively. The roughness of the bio composite surface was increased proportionally with the increased of composition of bacterial cellulose (BC) added to the bio composite samples. The morphology of the cross section was packed due to the increasing of bacterial cellulose (BC) composition in the bio composite. According to Martins et al. (2009), when the composition of bacterial cellulose (BC) increased, the strong interfacial adhesion between the cellulose fibers and the starch matrix occurred. However, Wan et al. (2009) state that the pores in the composites were obvious due to the increasing of bacterial cellulose (BC) and it not indicating a good fibre–matrix bonding.

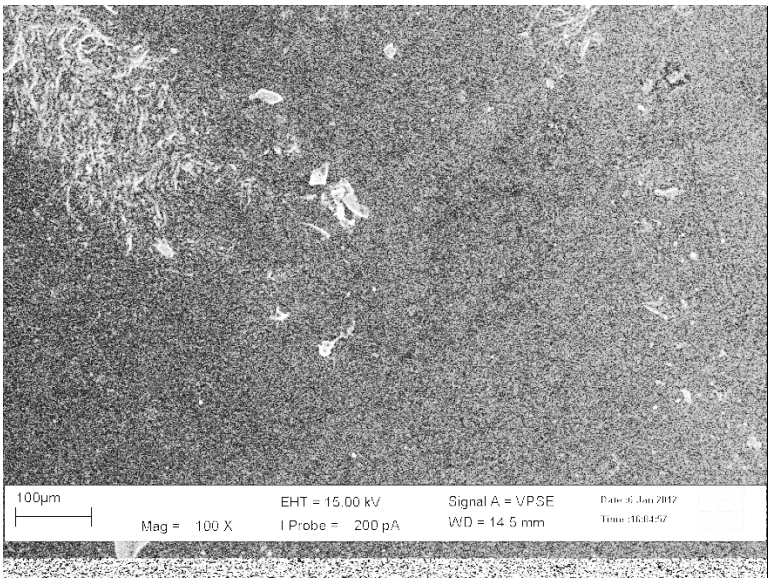

Figure 4.18: Scanning Electron micrograph of surface of 14BC+0% areca nut

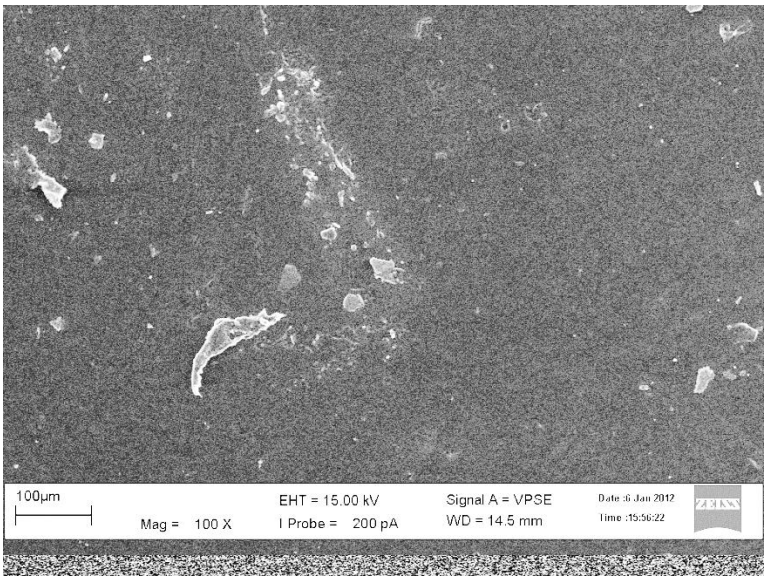

Figure 4.19: Scanning Electron micrograph of surface of 14BC+50% areca nut

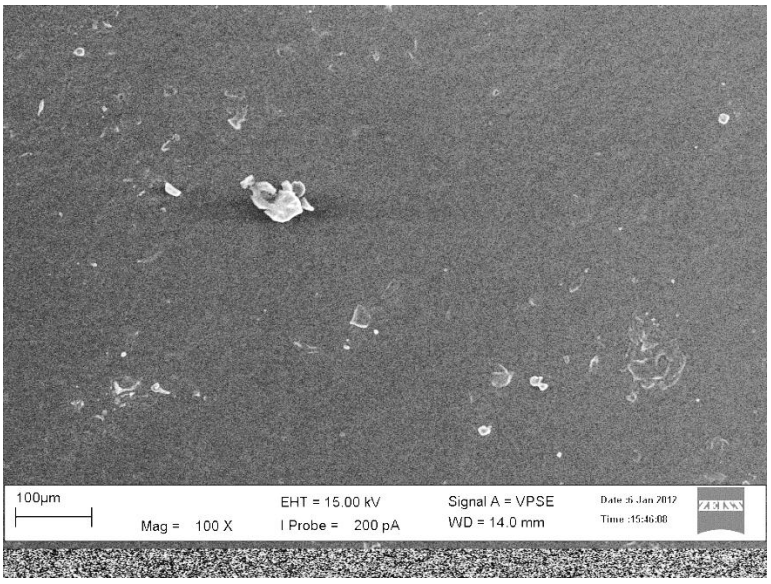

Figure 4.20: Scanning Electron micrograph of surface of 14BC+100% areca nut

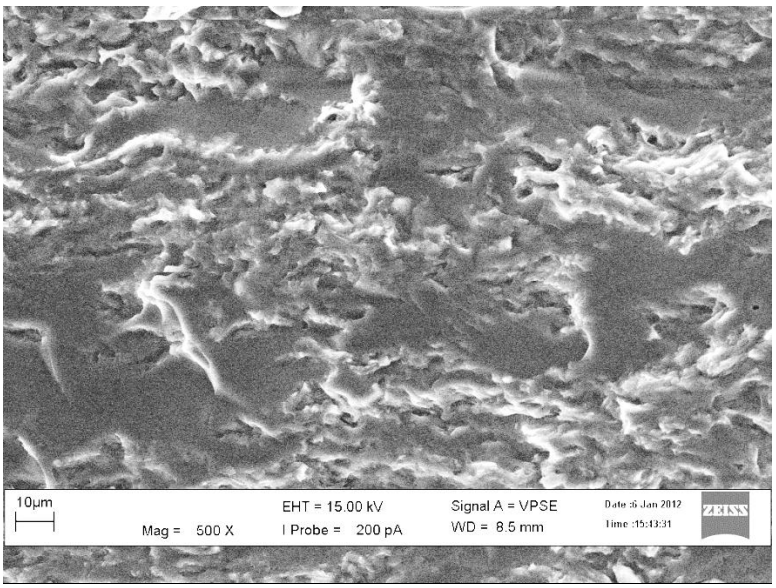

Figure 4.21: Scanning Electron micrograph of cross section of 14BC+0% areca nut

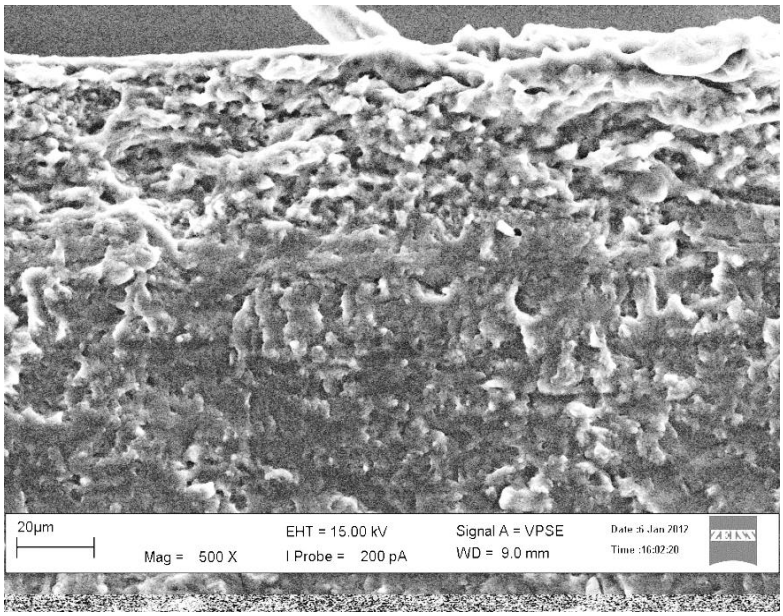

Figure 4.22: Scanning Electron micrograph of cross section of 14BC+50% areca nut

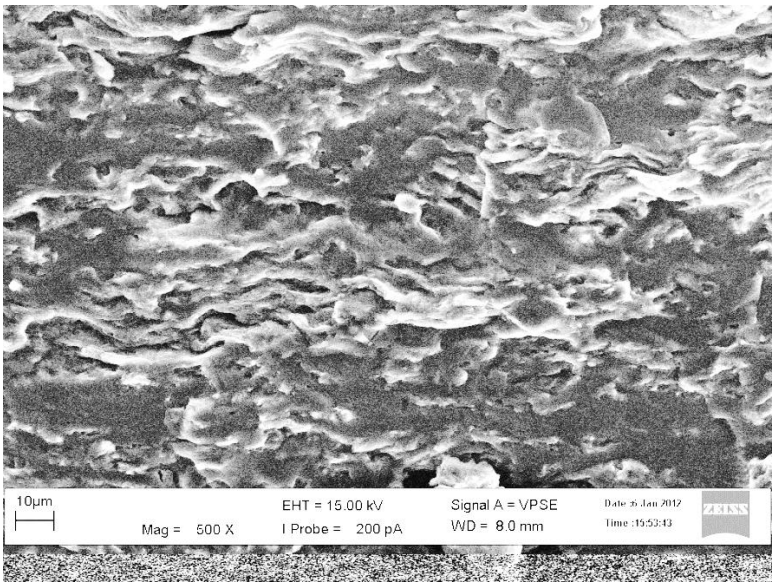

Figure 4.23: Scanning Electron micrograph of cross section of 14BC+100% areca nut

Figure 4.18 to Figure 23 presents the differences in surface and cross section micrographs of the bio composite samples with 14 grams of bacterial cellulose (BC) composition with 0%, 50% and 100% of areca nut extract, respectively. The surface of the bio composite samples become rougher when the percentages of areca nut extract was increased. According to Saibuatong and Phisalaphong (2010), the structures of the bio composite become less uniform due to the excessive on the film surface. Figure 4.10 (d-f) shows the cross section of each bio composite samples. The bio composite sample with 14 grams of bacterial cellulose (BC) and 100% of areca nut extract displayed good incorporation of areca nut extract into the bacterial cellulose (BC) fibril network due to the hydrogen bond between carbonyl group from cellulose and hydroxyl group from areca nut extract (Guzun et al., 2011). The structures of this bio composite sample were compact and well aligned than the other samples.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The production of antibacterial bio composite film can be produce from the mixing of bacterial cellulose (BC), starch glycerol and extraction of Areca nut with the additional of water by using the fabrication process. Analysis of antibacterial film from bacterial cellulose (BC) and *Areca Catechu* extract was done by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), soil degradable test, water absorption test, biodegradable by using fungi and testing for antibacterial activity by using Disc Diffusion assay, Quantitative of tannin and Phenolic Content Analysis.

From the result, the sample with the composition of 28 grams bacterial cellulose (BC) and 100% areca nut extract shows the optimum characteristic to be antibacterial film for wound healing. From the phenolic content test it the highest concentration of phenolic content and maximum zone of inhibition areas were observed when antibacterial testing was done by using *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*). Besides that, by adding the composition bacterial cellulose (BC) and areca nut percentage of water absorption and diffusivity of bio composite film will decrease. This is due to the presence of strong hydrogen bond from cellulose carbonyl group and phenolic hydroxyl group that contain in the film. The FTIR confirmed that bacterial cellulose (BC) and areca nut extract film contain the carbonyl group and hydroxyl group that used to form strong hydrogen bond.

The composite was shown the degradable characteristic. It was proved by soil degradable test and test of biodegradable by incubate the film with *Aspergillus niger* strain. The composite shows the low weight loss after the test because the content of areca nut extract has resistant to fungal attack. From all the test that have been done, it show that the bacterial cellulose (BC) and areca nut had potential to be used as wound healing because it can has antibacterial characteristic and contain phenol that can be used to treat wounds or injuries. Moreover, it also can be degrade and at the same time can protect the environment.

5.2 RECOMMENDATION

In order to improve the production of antibacterial film, more study should be done to investigate the effect when the film expose to the environment conditions. The film should be kept in the vacuum container to prevent it degrade when it expose to the extreme condition.

Extract of areca nut is main component to produce the antibacterial film. In order to get the high yield and high quality of extraction product, the fresh areca nut should be used to make sure the phenolic content in the areca nut do not degrade or decrease. For the extraction step, some improvement can be done by study the most suitable solvent that can be used to improve the extraction process. This step is important to make sure the areca nut can produce high yield of extract product.

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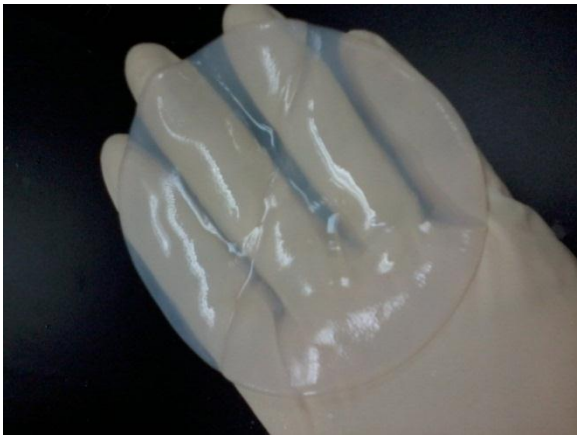
APPENDICES

APPENDIX A

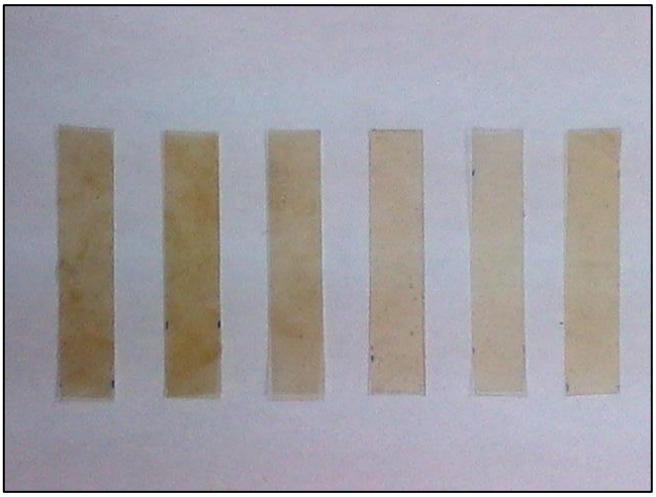
ANTIBACTERIAL BIO COMPOSITE PREPARATION AND TESTING

i. Bacterial cellulose (BC).

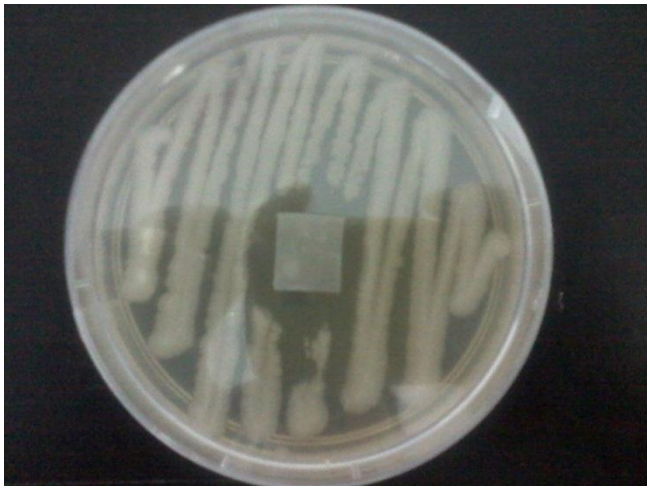


ii. Antibacterial bio composite (peeled from petri dish).

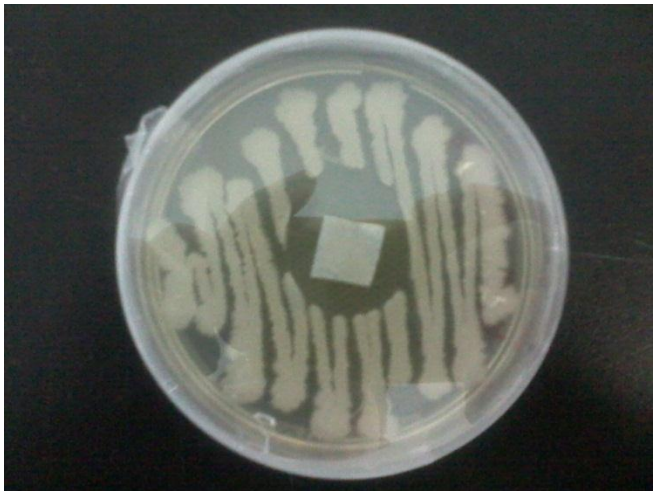
iii. Antibacterial bio composite.



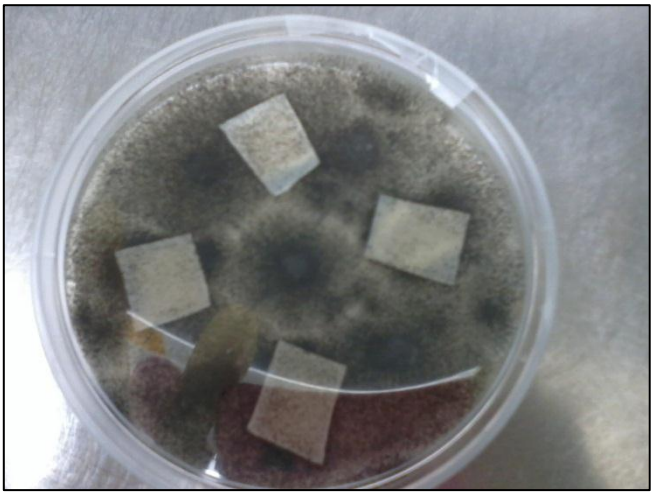
iv. Antibacterial test (*E. coli*).



v. Antibacterial test (*S. aureus*).

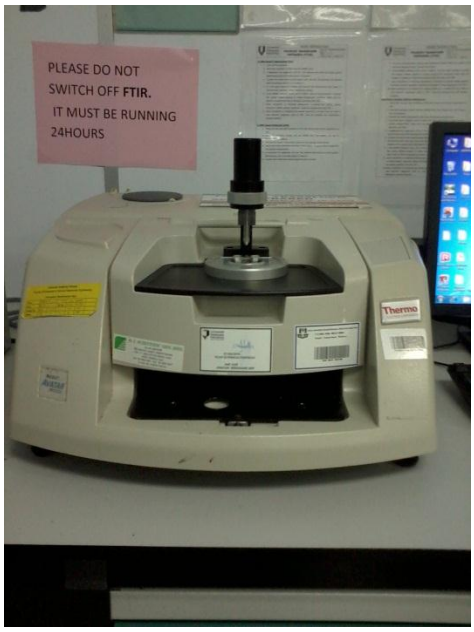


vi. Biodegradable test by using *Aspergillus niger.*



vii. Scanning Electron Microscope (SEM).

viii. Fourier Transform Infrared spectroscopy (FTIR).



APPENDIX B

DATA OF STANDARD CALIBRATION CURVE FOR PHENOLIC CONTENTS ANALYSIS

Table of preparation of Gallic acid for standard calibration curve

Concentration (mg/L)	Weight of Gallic acid was needed (g)	OD
100	0.01	1.4454
200	0.02	1.7454
300	0.03	2.0454
400	0.04	2.3454
500	0.05	2.6454

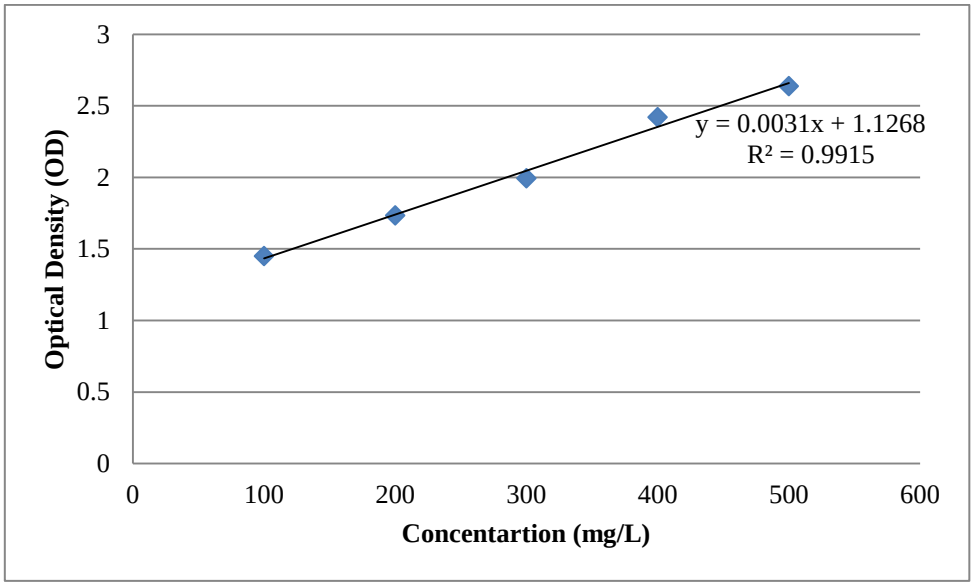


Figure of standard calibration curve for Phenolic contents analysis

APPENDIX C

DATA OF WATER ABSORPTION TEST

Table C1: Raw data for water absorption test (0% areca nut extract)

Time (min)	Weight (g)				
	0g BC	7g BC	14g BC	21g BC	28g BC
0	0.1207	0.1241	0.1216	0.1259	0.1192
10	0.1778	0.1851	0.1835	0.1835	0.1777
20	0.1907	0.1948	0.1902	0.1878	0.1785
30	0.1949	0.1987	0.1912	0.1882	0.1787
40	0.1997	0.2008	0.192	0.1885	0.1787
50	0.2022	0.2023	0.1927	0.1887	0.1787
60	0.2051	0.2027	0.1929	0.1887	0.1787
70	0.206	0.2029	0.1929	0.1887	0.1787
80	0.2061	0.2029	0.1929	0.1887	0.1787
90	0.2061	0.2029	0.1929	0.1887	0.1787
100	0.2061	0.2029	0.1929	0.1887	0.1787
110	0.2061	0.2029	0.1929	0.1887	0.1787
120	0.2061	0.2029	0.1929	0.1887	0.1787

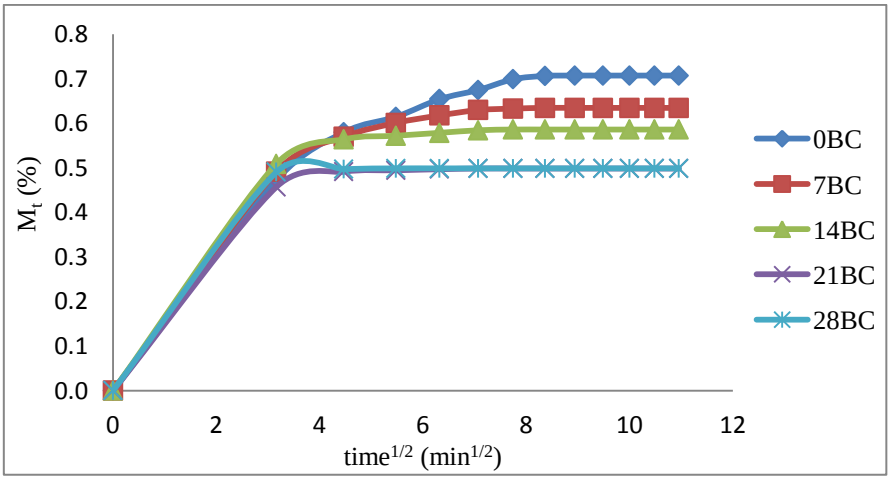


Figure C1: M_t versus $t^{1/2}$ curve (0% areca nut extract).

Table C2: Raw data for water absorption test (25% areca nut extract)

Time (min)	Weight (g)				
	0g BC	7g BC	14g BC	21g BC	28g BC
0	0.1223	0.1162	0.1183	0.1275	0.1244
10	0.1788	0.1701	0.1737	0.1816	0.1819
20	0.1911	0.1794	0.1814	0.1873	0.1831
30	0.1958	0.1821	0.1835	0.1878	0.1833
40	0.1984	0.1843	0.1842	0.1879	0.1833
50	0.2003	0.1847	0.1847	0.1879	0.1833
60	0.2016	0.1849	0.1848	0.1879	0.1833
70	0.2022	0.1851	0.1848	0.1879	0.1833
80	0.2026	0.1851	0.1848	0.1879	0.1833
90	0.2027	0.1851	0.1848	0.1879	0.1833
100	0.2028	0.1851	0.1848	0.1879	0.1833
110	0.2028	0.1851	0.1848	0.1879	0.1833
120	0.2028	0.1851	0.1848	0.1879	0.1833

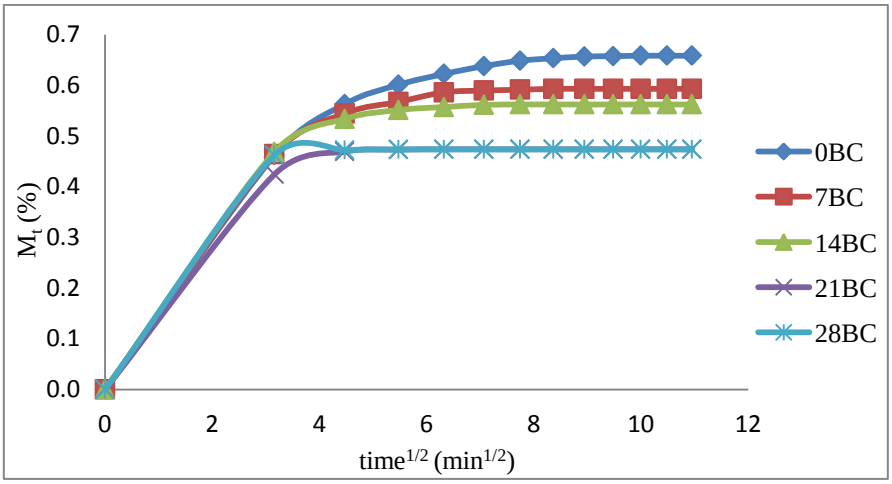


Figure C2: M_t versus $t^{1/2}$ curve (25% areca nut extract).

Table C3: Raw data for water absorption test (50% areca nut extract)

Time (min)	Weight (g)				
	0g BC	7g BC	14g BC	21g BC	28g BC
0	0.1174	0.1276	0.1231	0.1226	0.1255
10	0.1737	0.1866	0.1801	0.1783	0.1811
20	0.1813	0.1908	0.1819	0.1792	0.1812
30	0.1846	0.1922	0.1822	0.1793	0.1812
40	0.1862	0.1927	0.1824	0.1794	0.1812
50	0.1867	0.1927	0.1826	0.1794	0.1812
60	0.187	0.1928	0.1826	0.1794	0.1812
70	0.1873	0.1928	0.1826	0.1794	0.1812
80	0.1875	0.1928	0.1826	0.1794	0.1812
90	0.1875	0.1928	0.1826	0.1794	0.1812
100	0.1875	0.1928	0.1826	0.1794	0.1812
110	0.1875	0.1928	0.1826	0.1794	0.1812
120	0.1875	0.1928	0.1826	0.1794	0.1812

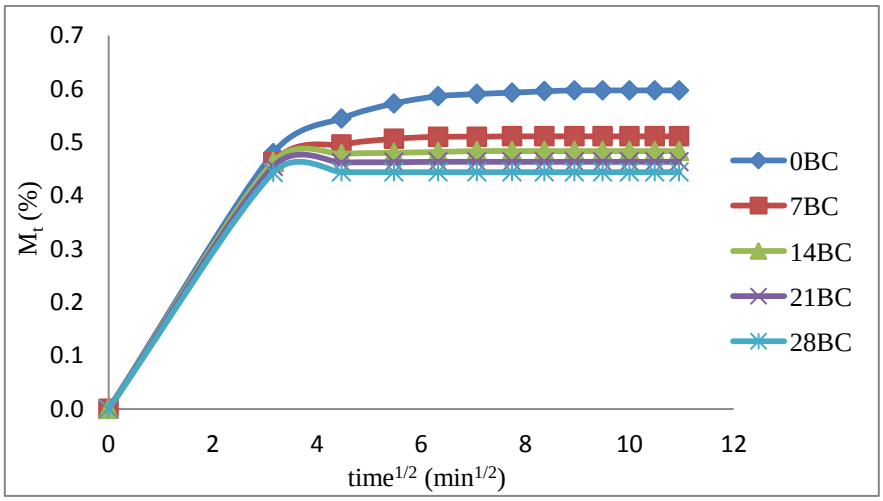


Figure C3: M_t versus $t^{1/2}$ curve (50% areca nut extract).

Table C4: Raw data for water absorption test (75% areca nut extract)

Time (min)	Weight (g)				
	0g BC	7g BC	14g BC	21g BC	28g BC
0	0.1172	0.1289	0.1228	0.1279	0.1238
10	0.1773	0.1876	0.178	0.1813	0.1757
20	0.1808	0.1912	0.1792	0.1819	0.1765
30	0.1832	0.1921	0.1804	0.1821	0.1765
40	0.184	0.1927	0.1805	0.1821	0.1765
50	0.1847	0.1931	0.1806	0.1821	0.1765
60	0.185	0.1933	0.1806	0.1821	0.1765
70	0.1852	0.1934	0.1806	0.1821	0.1765
80	0.1852	0.1934	0.1806	0.1821	0.1765
90	0.1853	0.1934	0.1806	0.1821	0.1765
100	0.1853	0.1934	0.1806	0.1821	0.1765
110	0.1853	0.1934	0.1806	0.1821	0.1765
120	0.1853	0.1934	0.1806	0.1821	0.1765

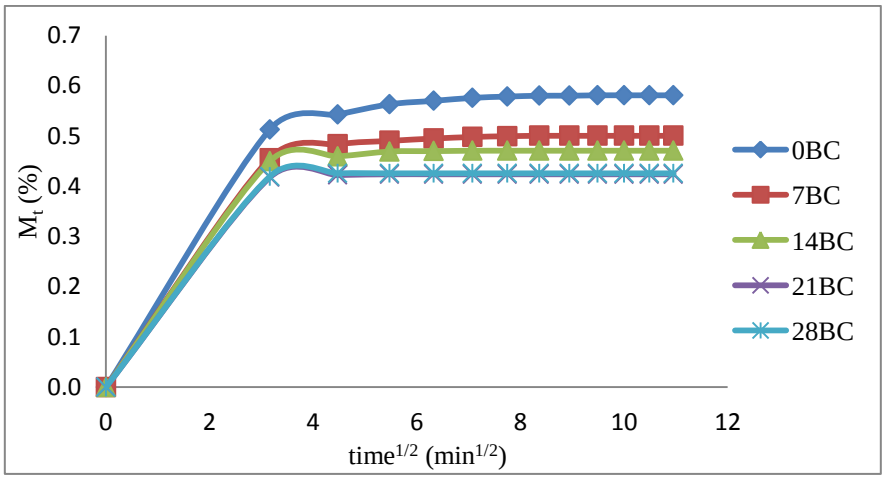


Figure C4: M_t versus $t^{1/2}$ curve (75% areca nut extract).

Table C5: Raw data for water absorption test (100% areca nut extract)

Time (min)	Weight (g)				
	0g BC	7g BC	14g BC	21g BC	28g BC
0	0.1210	0.1193	0.1203	0.1206	0.1298
10	0.1763	0.1709	0.1671	0.1627	0.1744
20	0.1813	0.1733	0.1687	0.1632	0.1754
30	0.1825	0.1742	0.1692	0.1636	0.1754
40	0.1832	0.1751	0.1693	0.1636	0.1754
50	0.1839	0.1756	0.1695	0.1636	0.1754
60	0.1843	0.1760	0.1695	0.1636	0.1754
70	0.1847	0.1762	0.1695	0.1636	0.1754
80	0.1849	0.1763	0.1695	0.1636	0.1754
90	0.1849	0.1763	0.1695	0.1636	0.1754
100	0.1850	0.1763	0.1695	0.1636	0.1754
110	0.1850	0.1763	0.1695	0.1636	0.1754
120	0.1850	0.1763	0.1695	0.1636	0.1754

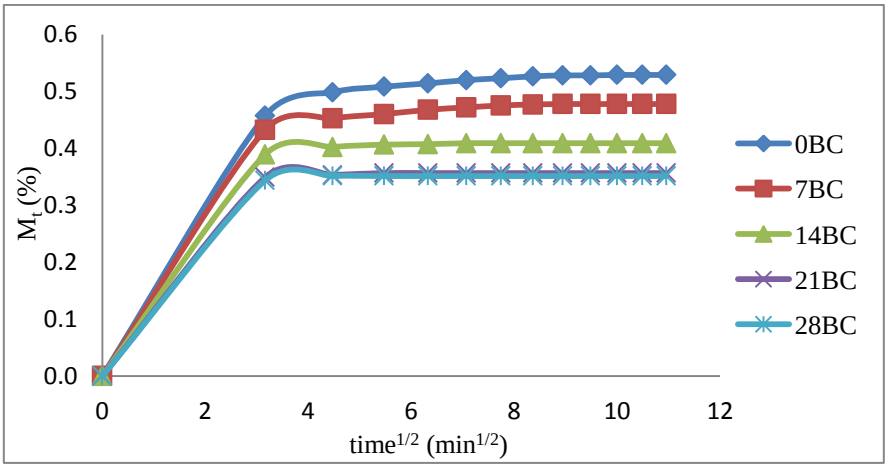


Figure C5: M_t versus $t^{1/2}$ curve (100% areca nut extract).

APPENDIX D

DATA OF SOIL BURIAL DEGRADABLE TEST

Table of bio composite samples weight per time

Areca nut extract	Bacterial cellulose (BC)	Day 0 (g)	Day 10 (g)	Day 20 (g)	Day 30 (g)
0%	0g	0.1802	0.1502	0.1183	0.0918
	7g	0.1764	0.1496	0.1264	0.1017
	14g	0.2763	0.2439	0.2074	0.1689
	21g	0.2407	0.2151	0.1839	0.1501
	28g	0.1926	0.1678	0.147	0.1254
25%	0g	0.2057	0.1775	0.149	0.1152
	7g	0.2667	0.2361	0.1989	0.1637
	14g	0.2619	0.2382	0.1974	0.1641
	21g	0.1946	0.1721	0.1521	0.1289
	28g	0.1893	0.1697	0.1549	0.1337
50%	0g	0.2191	0.1899	0.1608	0.1314
	7g	0.2072	0.1834	0.1589	0.1344
	14g	0.1883	0.168	0.1487	0.1276
	21g	0.1922	0.1734	0.1569	0.1353
	28g	0.1988	0.1831	0.1629	0.1453
75%	0g	0.1914	0.1692	0.147	0.1227
	7g	0.2264	0.2018	0.1794	0.1555
	14g	0.1446	0.1321	0.1188	0.1052
	21g	0.1544	0.1429	0.13	0.1181
	28g	0.1887	0.1752	0.1617	0.1482
100%	0g	0.1438	0.1294	0.1141	0.0995
	7g	0.1576	0.1429	0.1302	0.1175
	14g	0.223	0.2013	0.1845	0.1701
	21g	0.1705	0.1426	0.1297	0.1375
	28g	0.2122	0.1998	0.1873	0.1742

APPENDIX E

DATA FOR QUANTITATIVE OF TANNIN TEST

Table of volume of solution A and solution B

Bacterial cellulose (BC)	Areca nut extract	A (mL)	B (mL)	Concentration of tannin (mg/L)
0g	0%	0	0	0
	25%	70.2	34.6	59.8
	50%	118.5	37.1	136.8
	75%	166.0	35.6	219
	100%	239.8	35.1	343.9
7g	0%	0	0	0
	25%	80.6	36.7	73.7
	50%	123.6	35.9	147.4
	75%	177.3	35.2	238.7
	100%	249.7	33.9	362.5
14g	0%	0	0	0
	25%	85.0	34.9	84.1
	50%	132.0	35.6	161.9
	75%	185.7	35	253.2
	100%	267.3	36.3	388.1
21g	0%	0	0	0
	25%	95.3	36.9	98.1
	50%	140.1	35.8	175.2
	75%	196.0	34.6	271.1
	100%	275.7	36.2	402.3
28g	0%	0	0	0
	25%	103.2	36.9	111.3
	50%	148.2	35.8	188.9
	75%	214.2	37.2	297.3
	100%	288.9	36.6	423.9

APPENDIX F

DATA OF BIODEGRADABLE BY USING *ASPERGILLUS NIGER* TEST

Table of bio composite samples weight per time

Areca nut extract	Bacterial cellulose (BC)	Day 0 (g)	Day 10 (g)	Percentage weight loss (%)
0%	0 g	0.1190	0.0556	53.3
	7 g	0.1500	0.0745	50.3
	14 g	0.1450	0.0812	44.0
	21 g	0.1580	0.0959	39.3
	28 g	0.1290	0.0935	27.5
25%	0 g	0.1190	0.0589	50.5
	7 g	0.1410	0.0749	46.9
	14 g	0.1700	0.0997	41.4
	21 g	0.0970	0.0619	36.2
	28 g	0.0980	0.0749	23.6
50%	0 g	0.1040	0.0591	43.2
	7 g	0.1240	0.0721	41.9
	14 g	0.1230	0.0775	37.0
	21 g	0.1350	0.0916	32.1
	28 g	0.1720	0.1353	21.3
75%	0 g	0.1070	0.0659	38.4
	7 g	0.1560	0.0998	36.0
	14 g	0.1070	0.0708	33.8
	21 g	0.1140	0.0829	27.3
	28 g	0.1330	0.1081	18.7
100%	0 g	0.1180	0.0791	33.0
	7 g	0.0980	0.0684	30.2
	14 g	0.1110	0.0808	27.2
	21 g	0.1450	0.1095	24.5
	28 g	0.1080	0.0894	17.2

APPENDIX G

EQUATION AND CALCULATION

i. Water absorption test.

By using data from Table C5 (14 g BC and 100% areca nut extract), the diffusivity at t=0(M_t) and t=120min (M_m) were determined by using equation 3.2.

$$\frac{M_t}{M_m} = 1 - \frac{8}{\pi^2}\sum \frac{1}{(2n+1)^2} \exp\left\{\frac{-D(2n+1)^2 \pi^2 t}{h^2}\right\}$$

Equation 3.2 can be reduced to equation 3.3.

$$\frac{M_t}{M_m} = \frac{4}{\pi^{1/2}}\left(\frac{Dt}{h^2}\right)^{1/2}$$

Diffusivity can be calculated from the slope (k) of M_t vs $t^{1/2}$ curve (Figure C5).

$$D = \left(\frac{kh}{4M_m}\right)^2 \pi$$

$$D = \left(\frac{(0.0422)(0.042)}{4(0.4090)}\right)^2 \pi$$

$$D = 4.72 \times 10^{-6} mm^2/\min$$

ii. Quantitative of tannin.

By using data from Appendix E, the concentration of tannin was determined by using equation 3.4.

$$\text{Concentration of tannin} = \frac{\text{(A-B) x (g tannin/ml } KMnO_4\text{)}}{\text{ml of sample solution}}$$

Based on research conducted by Weteitayaklung et al. (2006), 1 ml of 0.04M of $KMnO_4$ = 0.00168 g of tannin (as gallotannic acid).

Concentration of tannin in bio composite (14 g BC and 100% areca nut extract)

$$= \frac{(267.3ml - 36.2ml) \times 0.00168g/ml}{1000ml}$$

= 388.1 mg/L

iii. Soil burial degradation test

By using data from Appendix D, the percentage of bio composite weight loss was determined by using equation 2.2.

$$\text{Weight loss (14 g BC and 100\% areca nut extract)} = \frac{W_o - W_t}{W_t} \times 100$$

$$= \frac{0.2230 - 0.1701}{0.1701} \times 100$$

= 23.7 %

iv. Biodegradable by using *Aspergillus niger* test.

By using data from Appendix F, the percentage of bio composite weight loss was determined by using equation 2.2.

Weight loss (14 g BC and 100% areca nut extract) = $\frac{W_o - W_t}{W_t} \times 100$

$= \frac{0.1110 - 0.0808}{0.0808} \times 100$

$= 27.2\ \%$