FABRICATION AND CHARACTERIZATION OF BACTERIA CELLULOSE, POLYVINYL ALCOHOL AND FRAGRANT OIL WITH ANTIMICROBIAL EFFECT FOR WOUND HEALING APPLICATION

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Thesis submitted in partial fulfillment of the requirements for the degree of

Bachelor of Chemical Engineering (Biotechnology)

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We declare that we have read this thesis and in our opinion this thesis is adequate in terms of scope and quality for the purpose awarding a Bachelor's Degree of Chemical Engineering (Biotechnology)

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I declare that this thesis entitled — "FABRICATION AND CHARACTERIZATION OF BACTERIA CELLULOSE, POLYVINYL ALCOHOL AND FRAGRANT OIL WITH ANTIMICROBIAL EFFECT FOR WOUND HEALING APPLICATION" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature: ……

Name : Mohd Syazwan Bin Shafie

Date : 1 February 2013

Dedicated, in thankful appreciation for support, encouragement and understanding to my beloved family, friends and my supervisor. May Allah s.w.t bless ourlife.

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ABSTRACT

The experiment is about the production of the wound healing product from the combination of three types of materials that is bacteria cellulose, polyvinyl alcohol (PVA) and also essential oil. The objective of the experiment is to fabricate the bacteria cellulose, polyvinyl alcohol (PVA) and essential oil with antibacterial properties for wound healing application. The method of the process can be divides into three that is preparation of bacterial cellulose, the fabrication of bacterial cellulose with PVA and essential oil and lastly characterization the product produced. In the fabrication process, the bacterial cellulose will be added with 1:4, 1:2, 3:4, and 1:1 ratios of PVA to BC. *Acetobactor Xylinum* is the bacteria that had been use to produce the bacteria cellulose. Among the method that will be used to characterize the product are Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), gas pycnometer, water absorption test and also antibacterial effect test. The entire tests are to make sure that the biocomposite film has the characteristics need as the wound healing material. The final product is in the form of film that is suitable to be used as the wound healing application. The result for the experiment is the higher ratios of PVA to BC content in the mixture of bacterial cellulose and essential oil will show the highest amount of the bond in the film. So, the present of the high amount of molecular bond inside the film will make it stronger. Then, for the SEM test, the higher ratios of PVA to BC content in the film will give the smooth morphology, homogenous and compact structure to the film. For moisture absorption test, the higher ratios of PVA to BC content will make the film can absorb more moisture compared to less amount of PVA content. For bacteria test, the higher ratios of PVA to BC content is more effective to kill more bacteria and lastly for the density test, the higher ratios of PVA to BC content in the film will give the higher density to the film. As the recommendation, use gram positive bacteria for the next study to see the effect of the biocomposite film with different ratio of PVA to BC content to the gram positive bacteria. Beside, try to produce the fragrant oil by yourself t to make sure the purity of the oil and also use

the combination of several fragrant oil to know the effectiveness of it antimicrobial effect.

FABRIKASI DAN PENCIRIAN SELULOSA BACTERIA, POLYVINYL ALCOHOL DAN MINYAK PATI DENGAN KESAN ANTIMIKROB UNTUK APLIKASI PENYEMBUHAN LUKA

ABSTRAK

Eksperimen ialah mengenai pengeluaran produk penyembuhan luka daripada gabungan tiga jenis bahan-bahan yang selulosa bakteria (SB), polyvinyl alkohol (PVA) dan juga minyak pati lavender. Objektif eksperimen adalah untuk menghasilkan selulosa bakteria, alkohol polyvinyl (PVA) dan minyak pati lavender dengan ciri-ciri antibakteria untuk penyembuhan luka. Kaedah pemprosesan terbahagi kepada tiga iaitu penyediaan selulosa bakteria, fabrikasi selulosa bakteria dengan PVA dan minyak pati dan diakhiri dengan pencirian produk yang dihasilkan. Dalam proses fabrikasi, selulosa bakteria akan ditambah dengan 1:4, 1:2, 3:04, dan 01:01 nisbah PVA terhadap SB. *Acetobactor xylinum* adalah bakteria yang telah gunakan untuk menghasilkan selulosa bakteria. Antara kaedah yang akan digunakan untuk mencirikan produk ialah Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy(SEM), piknometer gas, ujian penyerapan air dan juga ujian kesan antibakteria. Ujian keseluruhan adalah adalah untuk pastikan bahawa filem biokomposit mempunyai ciri-ciri yang diperlukan sebagai bahan penyembuhan luka. Produk akhir adalah dalam bentuk filem yang sesuai untuk digunakan sebagai bahan penyembuhan luka. Keputusan eksperimen menunjukkan nisbah kandungan PVA terhadap SB yang lebih tinggi dalam campuran selulosa bakteria dan minyak pati akan menunjukkan jumlah ikatan antara molecul yang tertinggi dalam filem. Jadi, kehadir jumlah yang ikatan molekul tinggi di dalam filem itu akan menjadikan ia lebih kuat. Kemudian, untuk ujian SEM, nisbah kandungan PVA terhadap SB yang lebih tinggi dalam filem akan memberi morfologi yang halus, struktur yang homogenus dan padat kepada filem. Untuk ujian penyerapan lembapan, nisbah kandungan PVA terhadap SB yang lebih tinggi akan membuat filem itu boleh menyerap lebih kelembapan berbanding dengan jumlah yang kurang kandungan nisbah PVA terhadap SB. Untuk ujian bakteria, nisbah kandungan PVA terhadap SB yang lebih tinggi adalah lebih berkesan untuk membunuh lebih banyak bakteria dan akhir sekali untuk ujian kepadatan, nisbah kandungan PVA terhadap SB yang lebih

tinggi dalam filem itu akan memberikan ketumpatan yang lebih tinggi kepada filem. Jesteru itu disyorkan penggunaan bakteria gram positif untuk kajian seterusnya bagi melihat kesannya terhadap filem biokomposit dengan nisbah kandungan PVA terhadap SB yang berbeza terhadap bakteria gram positif. Disamping itu, disyorkan juga untuk menghasilkan minyak pati secara sendiri bagi memastikan ketulenan minyak pati dan juga menggunakan gabungan minyak pati untuk mengetahui keberkesanan nya terhadap kesan antibajteria.

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LIST OF NOMENCLATURES

^{o}C	degree Celcius
BC	bacterial cellulose
CFU	colony forming units
EAA	Effectiveness antibacterial activity
FTIR	Fourier transform infrared spectroscopy
Mm	millimeter
µL	micrometer
PVA	polyvinyl alcohol
SEM	scanning electron microscopy

CHAPTER 1

INTRODUCTION

1.0 Background Of Study

Skin is the biggest organ of the human body. Its act as the barrier against the environment and also the first barrier of defend for human body from pathogen and infection. If the skin is damaged or destroyed, the moisture content and protein from the skin will be lost and will caused the infection to the damage area or wound to be increased. When the area of the wound increased, bacteria or pathogen can easily enter the human body that can caused internal infection. Healing of skin wound is complex process which requires the involvement of many different tissues, cell types and also matrix component (Martin *et al*., 2007; Balasubramaniam *et al*., 2001)

Wound dressing is the method use by person as the application of the wound healing. It usually used in the first aid and nursing. Wound dressing is different from bandage because bandage only function to make sure dressing is at its place. Dressing was design to be directly having contact with wound. Historically, wound dressing was

made by a piece of material such as cloth, cobwebs, dug, leaves and also honey. In the 1960s, that gauze and other absorbent materials (e.g. cotton) were passive products that "plugged and concealed" the wound but did little to encourage wound healing resulted in the creation of a minimal set of criteria for an ideal dressing (Turner 2001).Nowadays, modern wound dressings include gauze, film, foams, hydrogel, polysaccharide pastes, granules and beads. According to Turner (1979), the idea wound dressing need to have the ability to absorb exudates and toxic components from the wounds surface, maintain a high humidity at the wound dressing interface, allow gaseous exchange, provide thermal insulation, protect the wound from bacterial penetration, be nontoxic and also can be remove easily without trauma to the wound.

Bacterial cellulose or also known as biocellulose is the cellulose that produced by bacteria. It is usually been used traditionally in the food industries especially in producing *nata-de-coco*. This food product is native to Philippines and unconventional based on the fermentation of the coconut water (Jagannath *et al.*, 2008). The bacteria used in the production of the bacteria cellulose is the *Acetobacto xylinum* (Yamada *et al.*, 2000). This bacteria can extracellulary synthesized the cellulose into nano-sized fibril. The latest application of the bacteria cellulose used is as the material in the medical application. Due to their great characteristics such as good in mechanical properties, water sorption capacity, porosity, stability and also conformability, it's have been reported used as an artificial skin for the person that had extensive wound that cause by burns, artificial blood vessel (Klemm *et al*., 2005), tissue engineering of cartilage (Svensson *et al*., 2005), wound dressing (Legeza *et al*., 2004) and also wound healing (Czaja *et al*., 2006)

The fragrant oil or also known as the essential oil, aromatic oil, ethereal oil and also steam volatile oil. Fragrant oil have been made from plant from the process of distillation and it is concentrated and easily to volatile. Before, the uses of the fragrant oil are only for the fragrant and also the flavor. But the uses of it had expended into pharmaceutical industries with the introduction of aromatherapy that well known that can give relaxation to the practitioner because of its ability to stimulate the sense. Beside it also have the ability to prevent infection that have been cause by microorganism such as bacteria and virus because it's naturally have an antibacterial properties (Aromaweb, 2012).

Polyvinyl alcohol (PVA) is representative of a water soluble synthetic polymer. The raw material used in the manufacture of polyvinyl alcohol is vinyl acetate monomer and it's involving the process of polymerization of vinyl acetate followed by partial hydrolysis. It is odorless, tasteless, translucent, and nontoxic. Polyvinyl alcohol is excellence in film forming, emulsifying and adhesive. Beside polyvinyl alcohol also resistances to oil, grease and solvents. However all their properties are depend on the humidity. The physical properties and specific functional uses of the polyvinyl alcohol are depending on the degree of polymerization and the degree of the hydrolysis. Low temperature crystalized PVA has light transparency and nutrition permeability (Kobayashi *et al*., 1992).

Biocomposite are formed from the natural fibers that usually from plants. Cellulose is one of the bicomposite that formed from plant and consist of biodegradable fibers and natural fibers. Bacteria cellulose is one of the biocomposite that synthesized by micro-organism that has received the great deal of attention in recent decades (Eichhorn *et al*., 2009).The wide-ranging used of the biocomposite are include in the

production of biomedical composites for gene delivery, tissue engineering and bone tissue engineering application (Svensson *et al*,.2005; Bodin *et al*., 2005)

1.1 Problem Statement

According to Czaja *et al*., (2006), unlike others product on the market, bacterial cellulose has all the ideal characteristics that need by wound dressing properties such as lower in cost , able to donate moisture, prevent infection, biocompatible, flexible, conforms to almost body surface and can reduce paint. Based on that, we want to produce new wound healing product from synthesized of bacterial cellulose and also to provide the user with the most effective healing material that can fulfill the requirement for better wound healing process.

1.2 Objective

The objectives of the experiment is to fabricate the biocomposite from bacteria cellulose, polyvinyl alcohol and fragrant oil with antibacterial properties for wound healing application

1.3 Scope of Study

The scopes of study are

i. To produce the biocomposite film from bacteria cellulose, polyvinyl alcohol and fragrant oil.

ii. To characterize the biocomposite film by using FTIR, SEM and to analyze the antimicrobial effect and also water absorption.

iii. To test the effectiveness of the biocomposite film from bacteria cellulose, polyvinyl alcohol and fragrant oil to wound healing application.

1.4 Rational and Significant of study

The rational and significant of study are

i. To produce the wound dressing material that is low cost and very effective for wound healing application.

ii. To increase the uses of bacterial cellulose in biomedical application

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Wound dressing is very important process and knowledge in daily life. It shows how to deal with wound if it happens. Antibiotic, bandage and plaster are the common wound dressing that always been used by people. But the process may take long time and high cost and less effective (Czaja *et al.*, 2005). This research will focus on the fabrication of the biocomposite film by using bacterial cellulose, polyvinyl alcohol (PVA) and also fragrant oil with antibacterial effect.

2.2 Bacterial Cellulose

Cellulose is the main component of the plant cell wall. But there are some bacteria that can also produce cellulose and the cellulose produced are called biocellulose or bacterial cellulose. Bacterial cellulose is an organic compound with the formula (C_6 H_{10} O_5). *Acetobactar xylinum* is a gram negative bacterium which can produce cellulose with using glucose as a common substrate. The bacterial cellulose extracellular excretion can form aggregated fibrils which crystallize into ribbon and assemble into a cellulose mat called as a pellicle (Suwannapinunt *et al*., 2007).

There are several bacteria that can synthesize cellulose such as genera *Aerobacter, Acetobacter, Achromobacter, Agrobacterium, Alacaligenes, Azotobacter, Pseudomonas, Rhizobium,* and *Sarcina* (Jonas and Farah, 1998). An overview of bacterial cellulose producers is presented in Table 2.1.

Table 2.1 Bacterial cellulose producers

Genus	Cellulose structure
Acetobactar	Extracellular pellicle, Compose of ribbon
Achromobacter	Fibrils
Aerobacter	Fibrils
Agrobacterium	Short fibrils
Alcaligenes	Fibrils
Pseudomonas	Non distinct fibrils
Rhizobium	Short fibrils
Sarcina	Amorphous cellulose
zoogloea	Not well defined

(Source: Jonas and Farah, 1998)

However, the only species that can produce enough cellulose for commercial interest is *Acetobacter* and among all of the *Acetobacter* species, *Acetobacter xylinum* is extensively used in producing the cellulose. Cellulose can be produced by using the bacterium in the fermentation process of the coconut water with an N source. It can be produced in either in static or agitated condition. If its static condition is maintained, cellulose can be harvested as a pellicle while if it in agitated it form as aggregated particles. Bacterial cellulose produced by using *Acetobacter xylinum* at the air-liquid interface of coconut water is popularly known as *nata-de-coco* (Jagannnath *et al.*, 2008). This product based on coconut water is native to the Philippines and was firstly making locally in the year of 1949. Beside of using coconut water, many others substrate can be used such as cane molasses, beet molasses, pineapple skin, cheese whey permeate , high solid permeate in order to produce *nata-de-coco* (Keshk and Sameshima, 2006). The maximum thickness of cellulose was obtained at pH 4.0 with 10% sucrose and 0.5% ammonium sulphate concentration (Jagannnath *et al.*, 2008).

Because of their ultra-fine network structure, bacteria cellulose has high biodegradability and unique mechanical strange (Brown, 1992). This showed that bacterial cellulose advantage superior from plant cellulose. The chemical and physical properties of the bacterial cellulose are such as mechanical strange, crystallinity and hydrophobicity (Muenduen *et al.*, 2008).Because of the unique properties, bacterial cellulose has found of multitude of applications such as in paper industries, textile and food industries and as a biomaterial in cosmetic and medicine (Ring *et al.*, 1986).

In medical field, bacterial cellulose approved that it is very suitable for wound dressing process especially wound that cause by burn cases (Czaja *et al.,* 2006). This is because it makes the healing process become faster and less scaring. What make bacterial cellulose's is very effective for wound healing application is its water holding ability and water vapor permeability. The high water holding ability provides a moist atmosphere at the injury site, which is suitable in healing process, while the wicking ability allows sea page from the wound to be removed from the site. According to studies by Czaja *et al.* (2004), biocellulose membrane is fully compatible and manages to prevent the burn wound from excessive fluid loss, thus accelerating the entire process of healing.

Nanocomposite material from cellulose can be fabricate statically by synthesize the bacterial cellulose gel. From the studies of Yasuda *et al.* (2005) and Nakayama *et al.* (2004) the nanocomposite for biomedical application with improved mechanical strange can be synthesis by soaking the bacterial cellulose with polyacrylamide and gelatin solution. With owning the antibacterial property and hydrophilicity, they are promising material for wound healing application (Fayazpour *et al*., 2006). Moreover, BC-PVA nanocomposite has been developed for potential application of vascular implant (Charpentier *et al*., 2006). It is also suitable for soft tissue replacement and controlled released that promoted to be a good dressing and wound healing.

2.3 Polyvinyl Alcohol (PVA)

Polyvinyl alcohol is the water-soluble synthetic polymer. It has an excellent film, emulsifying and adhesive properties. Emulsifying means that it can change the complex substance into small droplet and then dissolve it. These properties make polyvinyl alcohol suitable to emulsify the bacterial cellulose film. It is also resistance to oil, grease and solvent. Beside, polyvinyl alcohol is odorless and nontoxic. The aroma barrier properties had made polyvinyl alcohol become odorless and nontoxic means it does not irritate when having contact with human skin. So, it not harmful and can be used to human skin safely. More than that, polyvinyl alcohol is fully degradable and dissolved quickly to make it as environmental free. Polyvinyl alcohol is hydrophilic, biocompatible, and flexible synthetic polymer (Ignatova *et al*., 2006; Alipour *et al.*, 2009 and Greiner, 2007)

PVA have been ideal candidates as biomaterial. This is due to their high degree of swelling, uncomplicated chemical structure, elastic in nature, nontoxic and non-carcinogenic, and also bioadhesive. Some of their application in biomedical are tissue reconstruction and replacements, cell entrapment and drug delivery, soft contact lens material, wound covering bandage for burn victims, and etc.(Hassan *et al*., 2000).

2.4 Fragrant Oil

Fragrant oil or essential oil is the concentrated volatile aromatic compound that produced by plant. Each drop of this precious liquid is extracted from many particular plant species that can be found in certain region on this world that growth in particular environment condition. The result is a very diverse type of aromatic smell that caused by their organic compound content.

Fragrant oils are extracted from many different part of plant that have oil sacs that is from the flower, leaves, stem, root, seed, wood and also bark depend to the species of plant. Essential oils can be extracted from plant organs by crushing or by distillation in a heated aqueous or alcoholic solvent, and their active components subsequently isolated and characterized using HPLC and gas-liquid chromatography.

Most of the essential oils have naturally antibacterial properties and varying of physical and emotional effect, such as stimulation, pain relief, relaxation and also healing. The uses of the fragrant oil by people have been for so long for various applications such as perfumes, flavors, and medicine. In the new modern era, fragrant oil has been used in perfumes, cosmetics, soaps, foods, confectionary, preservatives, insect repellents, oral health care and pharmaceutical product.

For medical application, because of many of fragrant oils possess antimicrobial, antiparasitic, anticancer and other medicine properties; they have been used in many medical field. Aromatherapy is one of the uses of fragrant oil that can stimulate the sense and give the relaxation to their practitioners. Besides, they also can fight infection, contain hormone-like compound, and initiate cellular regeneration and also act as chemical defense against fungal and viral. The medicinal properties of fragrant oil have

been increasing the attention over the past 20 to 30 years but still less than 10% of approximately 250000 of the world's flowering plant species have been analyzed for their pharmacological properties. Almost 25% of active, medical compounds currently prescribed in the USA and UK are isolated from higher plant (Anthony *et al.*, 2005).

Moreover, fragrant oil has a structure that similar to some compound that found in the blood and tissues. These make them compatible with human physiology. So, the uses of essential oils as the wound healing is very suitable with their properties where it can fight the infection as it is a naturally antimicrobial and also it the healing quality where it can initiate cellular regeneration. Besides that, fragrant oil also gives effect on this film because the properties of the oil itself are antimicrobial (Manuela *et al*., 2010).

2.5 Analysis Equipment

2.5.1 Fourier Transform Infra-Red (FTIR)

Fourier Transform Infra-Red (FTIR) is the analytical technique that has been used to obtain the infrared spectrum of absorption. The FTIR collect the data in the wide spectral range which measure the intensity of the narrow range of the wavelength at a time. The aim of the FTIR is to measure the effectiveness of the sample to absorb the light at each wavelength. Such element likes Si, O, H, C and N; all of them are absorb the light at the different wavelength. FTIR has been used to identify unknown materials

the quality or consistency of a sample and also determine the amount of components in a mixture (ThermoNicolet, 2001).

2.5.2 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) is a high resolution surface imaging technique and a type of electron microscope that using electron beam to scanning the sample. The electron then interacts with atom of the sample and produces the signal that will show the information about the structural topography of the sample, composition and also their properties. The types of signal that produced by SEM were secondary electrons, back-scattered electrons (BSE), transmitted electron, specimen current and X-ray. The Specimen that needs to use SEM normally required being completely dry. Many biological process and structures occur at surfaces and if antibodies are available, their components can be located within the surface structure. This is usually done in a similar way to immuno-fluorescence, using an unconjugated primary antibody followed by a tagged secondary antibody against the primary. A SEM can provide a wide range of magnification, from 30× to 100,000×, and the SEM chamber is large to work with (Li M *et al.*, 2005 and Liu *et al.*, 2009).

2.5.3 Gas Pycnometer

The gas pycnometer is one of the non-destructive techniques for density measurements. This device allows measurements of volume with high precision (accuracy of an order of 5×10−5). All types of pycnometers are recognized as density measuring devices. They are in fact devices for measuring volume only. Density is merely calculated as the ratio of mass to volume; mass being invariably measured on a discrete device, usually by weighing. The volume measured in a gas pycnometer is that amount of three-dimensional space which is inaccessible to the gas used, i.e. that volume within the sample chamber from which the gas is excluded. Therefore the volume measured considering the finest scale of surface roughness will depend on the atomic or molecular size of the gas helium therefore is most often prescribed as the measurement gas, not only is it of small size, it is also inert and the most ideal gas. According to Agnew and Leonard (2003), the density of sample also influences the mechanical properties such as strength, porosity, and ease of compaction.

2.5.4 Antimicrobial Testing

Antimicrobial test is done to demonstrate the growth of the bacteria and to measuring the antimicrobial susceptibility exist. The process followed the principle of diffusion or dilution of antimicrobial agent and these are available in a variety of formats (Walker, 2006). The test that usually use is quantitative test which can generate the

qualitative data. One of the quantitative tests is agar dilution method that uses the combination of dilution and agar diffusion.

The antimicrobial effect is most important test for that film. This is because this film must have an ability to stop the growth of bacterial. The polyvinyl alcohol-cellulose film demonstrated effective antimicrobial capability against *Escherichia coli* and *Staphylococcus aureus*. One of the ideal wound dressing properties are provide physical barrier against bacterial infection (Czaja *et al.*,2006). When the biocomposite film is able the infection of bacteria to the wound surface, the wound healing process will become more efficient.

2.5.5 Water absorption

Water absorption is the amount of water that move in or enter into the composite material when immersed into the water for certain period of time. Beside it is also the ratio of the water absorbed by material per weight of dry materials. This test was designed to evaluate materials by exposing specimens to water for different time & temperature profiles. Testing is conducted on specimens that are submersed in water and a before & after weight change is documented. Depending on the application of the product, certain levels of moisture are accepted (crtlabs, 2012).

To get the effective composite material, the material must resist moisture. So, the bacterial cellulose, polyvinyl alcohol and fragrant oil film should resist the moisture movement from inside and outside the film. As an ideal properties of wound care

dressing, they are must be able to create and maintain a moist environment in the wound for more effective healing process. (Czaja *et al.*, 2006)

CHAPTER 3

METHEDOLOGY

3.1 Introduction

This chapter detail on the procedure used for this research to produce healing product of wound for antimicrobial effect by using bacteria cellulose, polyvinyl alcohol and also fragrant oil. First process is to prepare the bacterial cellulose by culturing (*Acetobactor xylinum*) in the culture medium. The next step is the fabrication of the bacterial cellulose with polyvinyl alcohol and also fragrant oil. Then the healing product were characterized by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscopy (SEM), gas pycnometer and to analyze the antibacterial effect and water absorption.

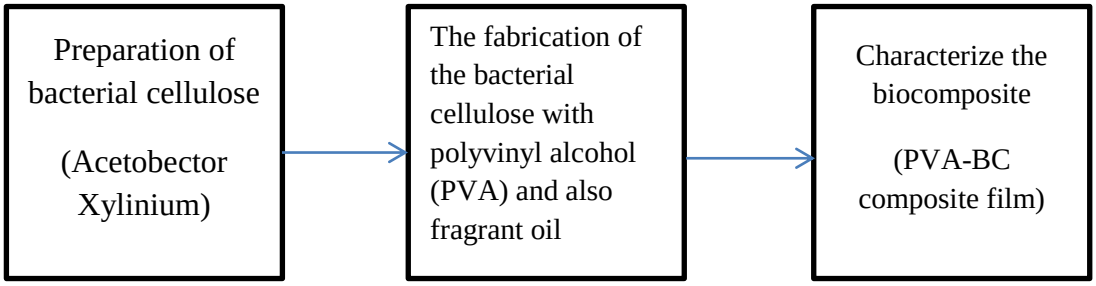

Figure 3.1 Overall process of fabrication of healing product.

3.2 Materials and Methods

3.2.1 Material

Polyvinyl alcohol (PVA), fragrant oil (lavender oil) purchased from Body Shop (Body Shop grade), while *Acetobactor xylium* bacteria *(A.xylium)* will be purchased from The Department of Microbiology, MARDI, Serdang, Malaysia.

3.2.2 Methods

3.2.2.1 Bacterial cellulose production

The culture medium for the inoculums was the coconut-water containing 80g sucrose and 5g ammonium sulfate. The medium was autoclaved at the temperature 121°C for 20 min. Then, the medium was leaved for a while for cooling process to take

place. Then checked the pH of the medium and if the medium pH more than 4.5 use the sodium hydroxide to get the required pH value. the After that, 25 mL of a stock culture was inoculating into 2500 mL of medium and then incubate at 30°C for 7 days in static culture. After 7 days, a brown layer (bacterial cellulose) was formed at the surface of the medium. Then, the bacterial cellulose was harvested and purified. The process of harvested and purification was started by removing the bacterial cellulose from the culture medium and washed it with deionized water. In order to remove the bacteria and eliminate the remaining culture medium, the bacterial cellulose film was treated in 2.5% NaOH at room temperature for 24 hour and then followed by treated with 2.5% NaOCl and lastly repeated rinsing with deionized water. The bacterial cellulose was stored in deionized water until it was used.

3.2.2.2 Film preparation

The polyvinyl alcohol solution was prepared by dissolving PVA according to the ratio of PVA to BC that is 1:4, 1:2, 3:4 and also 1:1 in the 50 ml distilled water. The composition of biocomposite films were showed in Table 3.1. The mixtures was heated and stirred on hot plate at temperature of 85 ± 2°C for about 20 minutes until it completely dissolved. Then, 3g of blended bacterial cellulose was added into the polyvinyl alcohol solution at 80°C. Then, the solution was mixed gently by stirred it with a magnetic stir bar until the solution becomes homogenize and concentrated. After the mixture look concentrated enough then put the lavender oil about 250 µl. Stirred the mixture for a while and casted it in the plastic petri dishes. Lastly, the mixtures were

dried at 65°C for 48 hours. After cooling down, the complex films from the petri dishes was removed and stored in the desiccators at room temperature.

Table 3.1 Composition of biocomposite films.

Bacteria Cellulose (BC)	Polyvinyl alcohol (PVA)	water	Fragrant oil	Ratio PVA:BC
3g	0.75g	50ml	250µl	1:4
3g	1.5g	50ml	250µl	1:2
3g	2.25g	50ml	250µl	3:4
3g	3g	50ml	250µl	1:1

3.2.2.3 Antimicrobial Testing

3.2.2.3.1 Disk Diffusion Method

The antibacterial activity of the sample film was tested using the bacteria *Escherichia coli (E.coli).* The agar plate for E.coli ware prepared using the nutrient agar. The samples use for the antibacterial assay was sterilized first at temperature 121°C by an autoclave over a period of 30 min. In the bacterial assay, 5cm x 5cm biocomposite film was placed on the top of nutrient agar containing of *E.coli*. Then, it was incubated at temperature 37°C for 24 hour. The inhibitory activity was measured based on clear zone surrounding on biocomposite films.

3.2.2.3.2 Dilution and Spread Plate Technique

The biocomposite films were prepared by 2cm x 2cm size. The biocomposite film then was introduced into a test tube that containing 9 mL nutrient broth and 1 mL bacterial culture. After that, the test tubes containing the mixture were incubated at 37°C for 24 hours. The bacteria that used for test were *E.coli.* After 24 hours, 1 ml of the mixture was added into 9 mL of distilled water. From diluted solution, the surviving bacteria were counted by spread plate method. Some of solution was transferred to agar media by using the loop rod and it's was inoculated at 37°C for 24 hours. The number of colony forming units, CFU, on plate was counted. The effectiveness of this film for antibacterial activity (EAA) was calculated according to Equation 3.1.

$$EAA = \frac{No-Ns}{No} \times 100\% \quad (3.1)$$

Where the No represents the number of bacteria with uncoated film in the plate and Ns represents number of bacteria with coated film in the plate.

3.2.2.4 Fourier Transforms Infrared Spectroscopy (FTIR)

Record the Fourier transform infrared (FTIR) spectrum on Nicolet Avatar 370 DTGS. The FTIR generates an infrared spectral scan of samples that absorb infrared light. Then, the FTIR spectra of the films were determined at wave number ranging from 800 to 3600 cm^{-1}.

3.2.2.5 Scanning Electron Microscopy (SEM)

The morphologies of the surface and the cross-section of the films was observed by using Scanning Electron Microscopy **(**SEM EDX Spectrometer EVO 50), operating at an acceleration voltage of 15kV. The surface of the biocomposite films was coated with gold under vacuum for SEM observation.

3.2.2.6 Density

3.2.2.6.1 Experimental

The biocomposite films were cut into 2 cm x 2 cm size and the initial weight ware measured as W0. After the biocomposite films were immersed in the distilled water around 15 min, the new weight was denoted as W1. The specific gravity of the solid is the ratio of its weight initial (W0) to the difference between its weight initial and its weight immersed in water (W0 - W1) as in Equation 3.2 and Equation 3.3.

$$Specific\ gravity\ (SG) = \frac{Wo}{(Wo - W1)} \tag{3.2}$$

$$Specific\ gravity\ (SG) = \frac{Density}{Density\ reference\ (water)} \tag{3.3}$$

3.2.2.6.2 Gas Pycnometer

The density of the biocomposite films were determined by using gas pycnometer (Micrometer model Accupyc II 1340). The films were put into the column for the analyzing and the weight of the film must ¾ of the weight column.

3.2.2.7 Water Absorption

The films were dried at 55°C in an oven for 15 min. After drying, the weight of film was measured as initial weight (W0). Then, the films were immersed in distilled water. Every 10 min the film was weight until the weight was constant. The mass gain at any time, (t) was determined and Mt as a result of moisture absorption by Equation 3.4.

$$Mt = \frac{(Wt - Wo)}{Wo} \tag{3.4}$$

Where Wo and Wt were denoted respectively as the weight of dry film (the initial weight of materials prior to exposure to the water absorption) and weight of film after exposure to water absorption.The rates of water absorption were obtained by using the Fick's law of diffusion. At an early stage, the diffusion process is presented in Equation 3.5.

$$\frac{Mt}{M\infty} = 2\left(\frac{Dt}{\pi L^2}\right)^{1/2} \tag{3.5}$$

Where Mt is the mass gain at time t, M∞ is the mass gain at equilibrium (hereafter, maximum water uptake), L is the thickness of the film and D the diffusion coefficient. The data was plotted as Mt/M∞ against $t^{1/2}$, and the diffusion coefficients was obtained from the slopes of the straight parts.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

This chapter detailed about the result and analysis for this research. The result would be based on the comparison between the previous researches that had been done before.

4.2 Biocomposite Film

The result of the fabrication of biocomposite film from bacteria cellulose, polyvinyl alcohol (PVA) and also fragrant oil are as shown below. The differences between the films with the different content of PVA can be seen base on its surface look. It can be seen that from the Figure 4.1, the surface structure of film are roughest

compared to other four films. Figure 4.2, 4.3 and 4.4 showed the decreases in the smoothness of the biocomposite film surface. Besides, the present of the white spot on the surface of the film are clearly seem on the surface of the film with ratio of 3:4 and 1:1 PVA to BC content.

From the observation, the film in Figure 4.2, 4.3 and 4.4 are not flat in shape as the film in Figure 4.1. The shape looks like to roll. The reason behind this situation is the present of higher ratio of PVA to BC in the film that reduce the time of the film to dry. To make sure this situation not occur during the fabrication of PVA, the heat of the incubator can be lowered or collect the sample with high content of PVA earlier that the lowest PVA content of film.

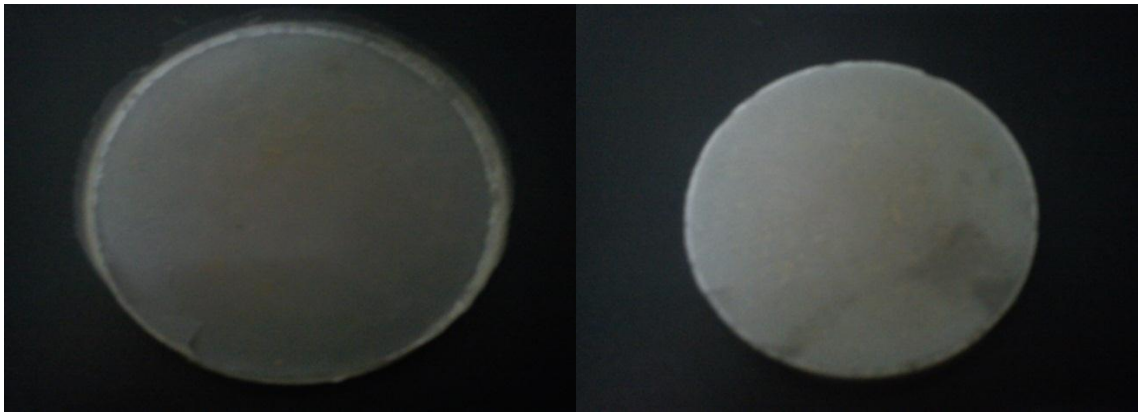

Figure 4.1 Structure of the upper surface and lower surface of biocomposite film with 1:4 ratio of PVA to BC.

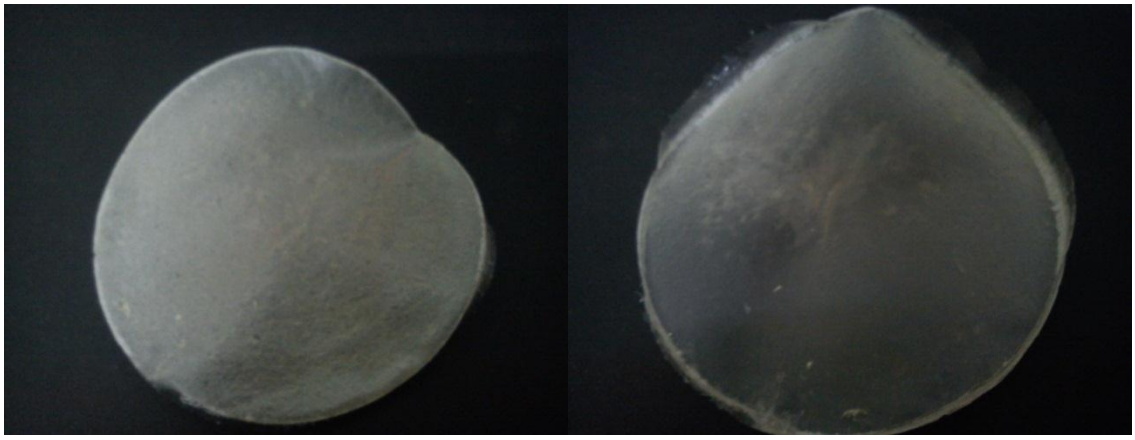

Figure 4.2 Structure of the upper surface and lower surface of biocomposite film with 1:2 ratio of PVA to BC.

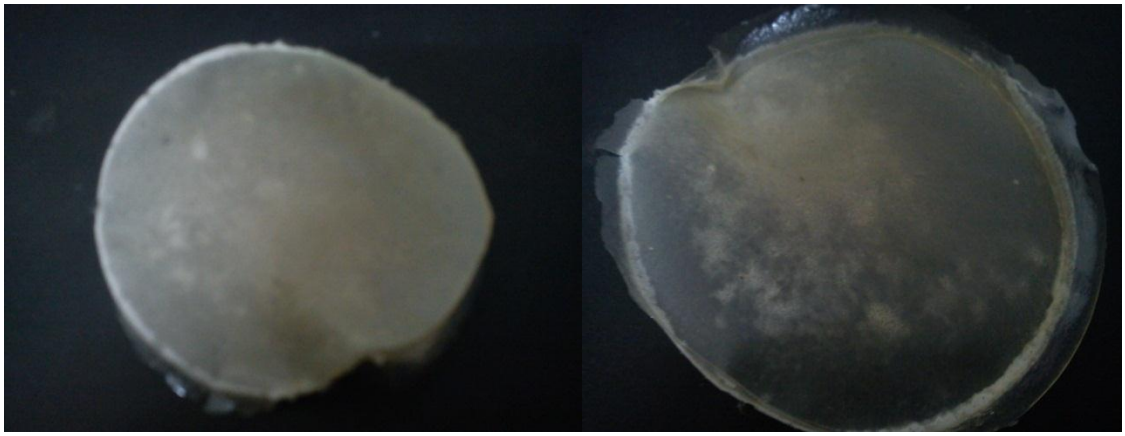

Figure 4.3 Structure of the upper surface and lower surface of biocomposite film with 3:4 ratio of PVA to BC.

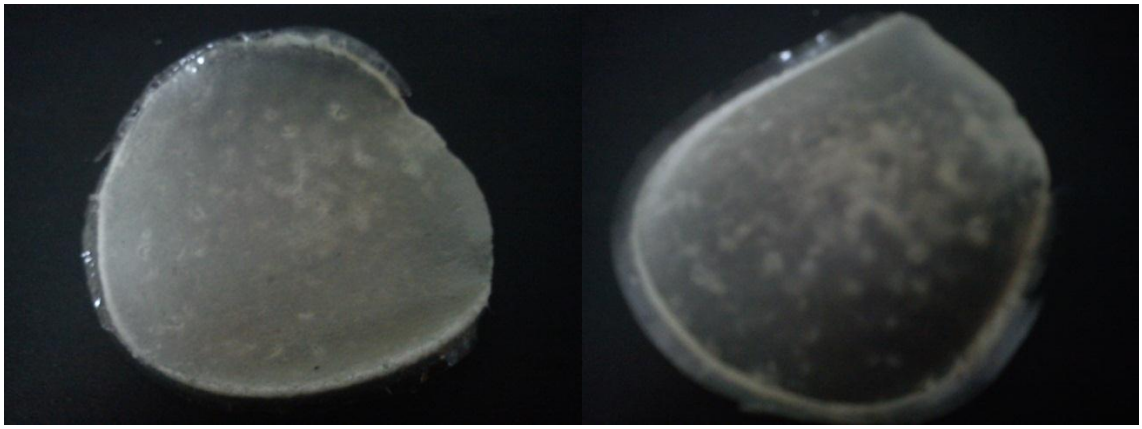

Figure 4.4 Structure of the upper surface and lower surface of biocomposite film with 1:1 ratio of PVA to BC.

4.3 Antimicrobial Testing

Antibacterial activities of the film against *E.coli* were explored by disk diffusion method and dilution and spread technique. Four different ratio of PVA to BC contents were used in this test. The capability of the film to inhibit the growth of tested bacteria was shown in Table 4.1. The results from the disk diffusion method are shown Figure 4.5. The high antibacterial activity can be found for the film that had 1:1 ratio of PVA to BC. The result of the disk diffusion test against *E. coli* was about 0.0023mm after four days. This showed the increasing patter of inhibition area of bacteria with the increases of the ratio of PVA to BC content in the film.

The second method used for determination of antibacterial activity was the dilution and spread plate technique. It describes the activity of a film in liquid medium. This method offers a relatively higher sensitivity and accuracy compared to the measurement of inhibition zones diameters. From Figure 4.6, it showed that the film was effective to kill the *E. coli*. From the results, indicated that the higher ratio of PVA to BC will increase the effectiveness of film to kill the bacteria. However the increasing in the effectiveness was low because of the BC and PVA were not contained the antimicrobial properties. The antimicrobial properties were only come from lavender oil. Fragrant oil gives effect on this film because the properties of the oil itself are antimicrobial (Manuela *et al.,* 2010). The present of lavender oil in the biocomposite film will improve the properties of the film itself against the bacteria. The fix amount of lavender oil used makes the different between the effectiveness of biocomposite with films were small. Same goes to the inhibitory zone reading for disk diffusion method test where the different in reading between the samples are too small.

The effect of the film towards *E.coli* bacteria was caused by the *E.coli* cell membrane. The *E.coli* bacteria made up from a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipid. Because of the bilayer structure of outer membrane at the *E.coli* it was become a potential barrier against foreign molecules comes into the cell (Wenming *et al.*, 2002).

4.3.1 Disk Diffusion Method

Table 4.1 Inhibitory zone of *E. coli* with different ratio of PVA to BC content

Sample (ratio PVA to BC content)	Inhibitory zone (mm)			
	Day 1	Day2	Day3	Day4
1:4	0.0000	0.0005	0.0013	0.0015
1:2	0.0000	0.0009	0.0014	0.0018
3:4	0.0003	0.0012	0.0017	0.0020
1:1	0.0005	0.0014	0.0019	0.0023

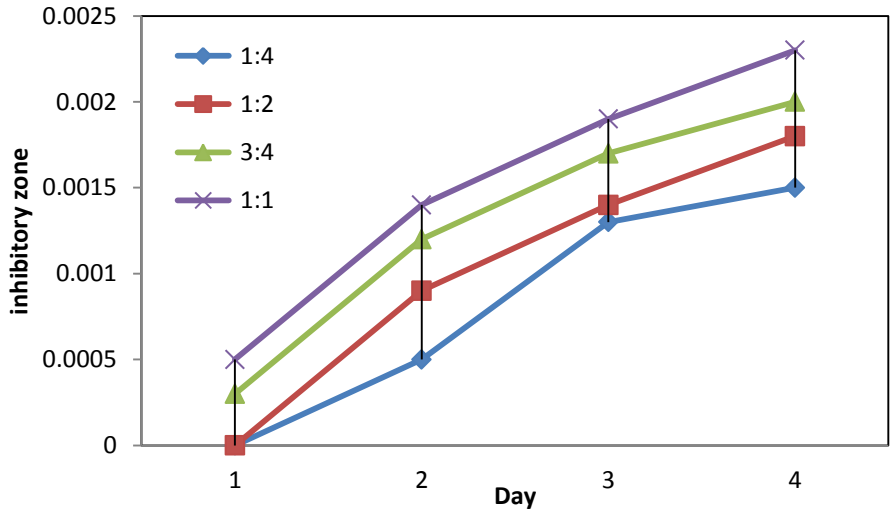

Figure 4.5 Inhibitory zone of *E. coli* at different ratio of PVA to BC content.

4.3.2 Dilution and Spread Plate Technique

Table 4.2 Percentage of effectiveness of different ratio of PVA to BC content against bacteria *E.coli*

Sample (Ratio of PVA to BC content)	Type of bacteria (percentage of effectiveness)
	E. coli
1:4	45.08%
1:2	45.43%
3:4	45.97%
1:1	46.03%

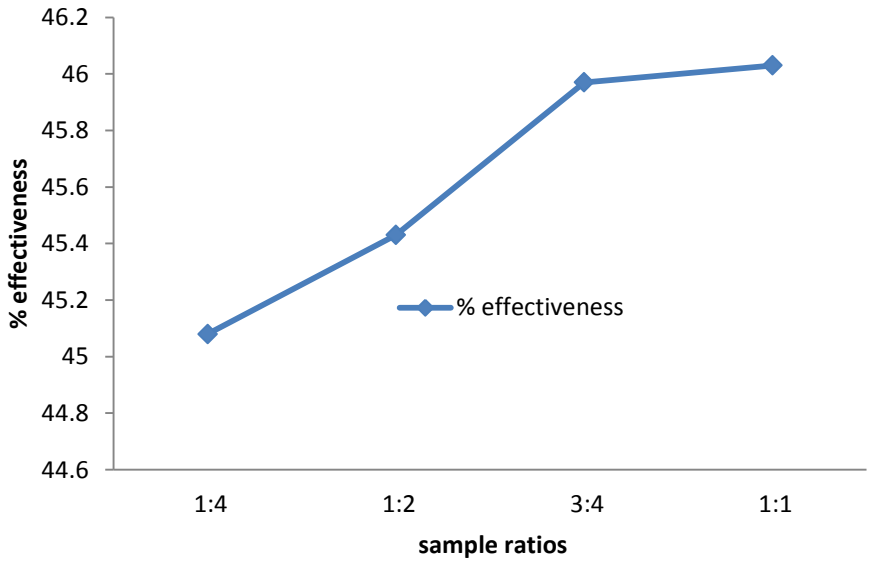

Figure 4.6 Percentage effectiveness of different ratio of PVA to BC content against bacteria *E.coli*.

4.4 Fourier Transforms Infrared Spectroscopy (FTIR)

FTIR was often used as a tool in determining the specific functional groups or chemical bonds in the sample. The samples that have been used were the film that fabricated from bacterial cellulose, PVA and lavender oil. The presence of a peak at a specific wave number would indicate the presence of a specific chemical bond. The infrared spectra of film were shown in Figure 4.7, 4.8, 4.9 and also 4.10 in wavelength range between 3,500-500 cm^{-1}.

In Figure 4.7, the characteristic band of biocomposite film with 1:4 ratio of PVA to BC content appeared at 3332.29 cm^{-1} for the hydroxyl group stretching vibration, at 2917.43 cm^{-1} for C-H stretching vibration, at 2287.42 cm^{-1} for C=C stretching vibration, at 1663.30 cm^{-1} and 1631.46 cm^{-1} also for C=C stretching vibration, at 1438.56 cm^{-1}, 1245.04 cm^{-1}, 1097.37 cm^{-1} and 1060.63 cm^{-1} for C-O stretching vibration and at 832.60 cm^{-1},743.26 cm^{-1},655.77 cm^{-1} for C-H bending vibration.

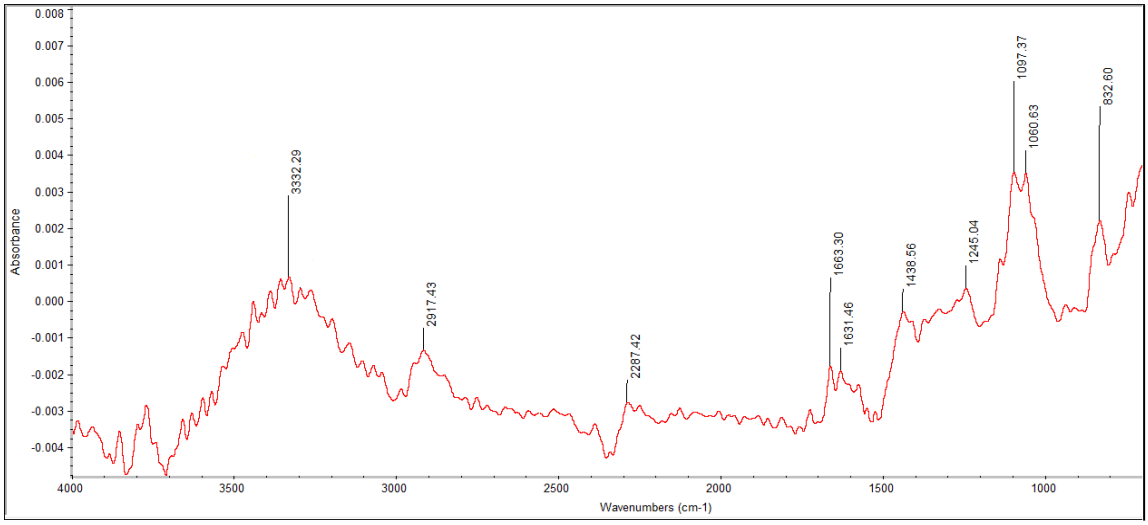

Figure 4.7 The infrared spectra of biocomposite film with 1:4 ratio of PVA to BC content.

In Figure 4.8, the characteristic band of biocomposite film with 1:2 ratio of PVA to BC content appeared at 3297.79 cm^{-1} for the hydroxyl group stretching vibration, at 2921.44 cm^{-1} and 2854.41cm^{-1} for C-H stretching vibration, at 1727.50 cm^{-1} for C=O stretching vibration, at 1654.14 cm^{-1} and 1544.78 cm^{-1} for C=C stretching vibration, at 1418.16 cm^{-1}, 1329.38 cm^{-1}, 1266.67 cm^{-1}, 1140.45 cm^{-1} and 1094.12 cm^{-1} for C-O stretching vibration and at 917.58cm^{-1} 842.30 $cm^{-1}$for C-H bending vibration.

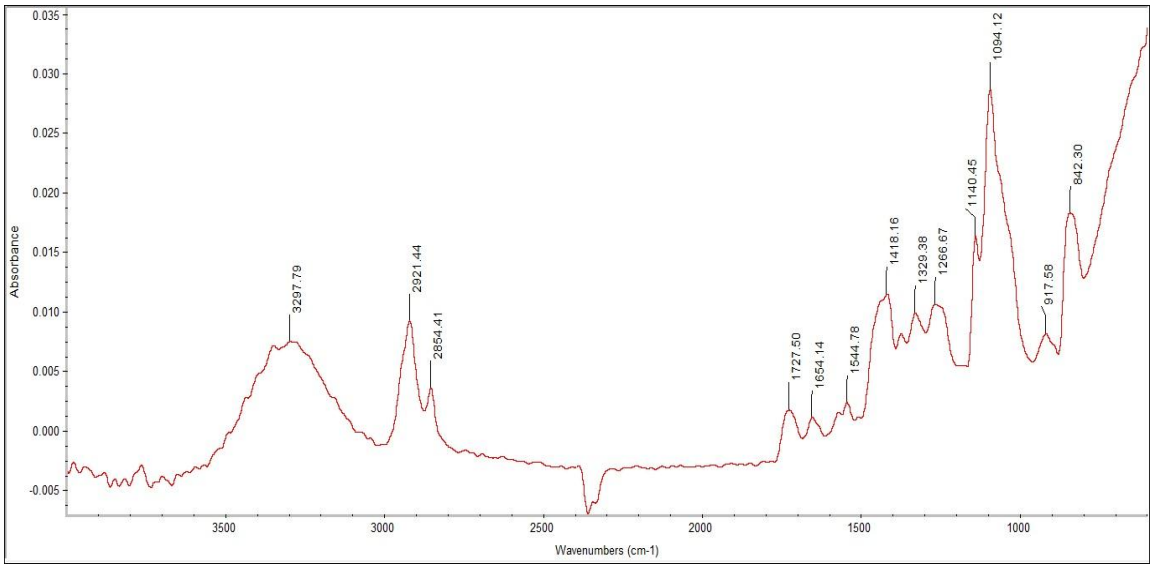

Figure 4.8 The infrared spectra of biocomposite film with 1:2 ratio of PVA to BC content.

In Figure 4.9, the characteristic band of biocomposite film with 3:4 ratio of PVA to BC content appeared at 3299.46 cm^{-1} for the hydroxyl group stretching vibration, at 2921.54cm^{-1} for C-H stretching vibration, at 2363.30 cm^{-1} for C=C stretching vibration, at 1652.87 cm^{-1} and 1544.49 cm^{-1} for C=C stretching vibration, at 1420.79 cm^{-1}, 1330.66 cm^{-1}, 1268.29 cm^{-1},1140.81 cm^{-1} and 1094.55 cm^{-1} for C-O stretching vibration and at 843.35 cm^{-1} for C-H bending vibration.

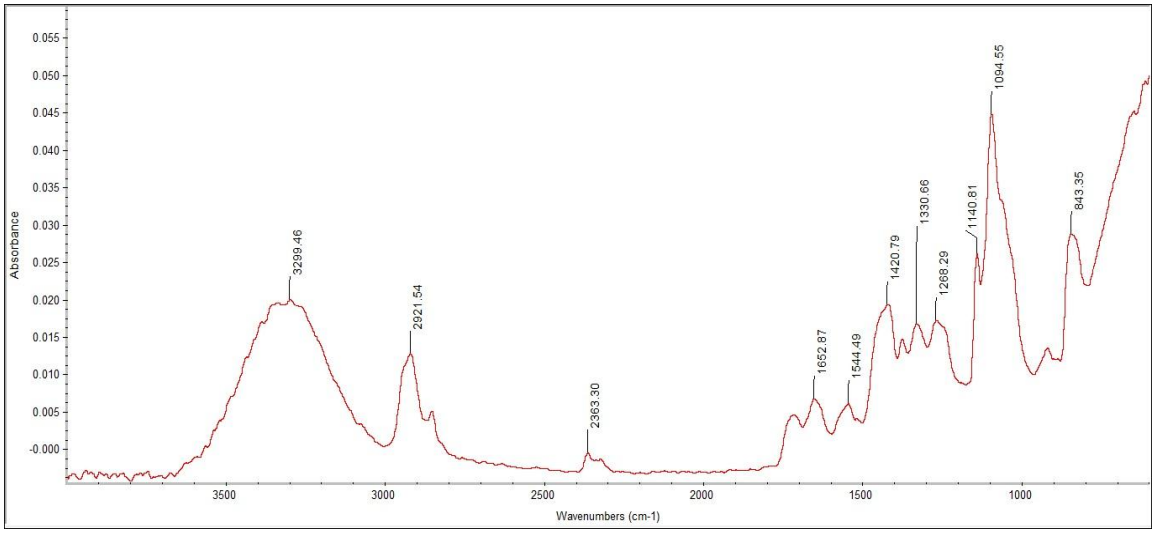

Figure 4.9 The infrared spectra of biocomposite film with 3:4 ratio of PVA to BC content.

In Figure 4.10, the characteristic band of biocomposite film with 1:1 ratio of PVA to BC content appeared at 3355.57 cm^{-1} for the hydroxyl group stretching vibration, at 2922.63 cm^{-1} and 2653.31cm^{-1} for C-H stretching vibration, at 1727.16 cm^{-1} for C=O stretching vibration, at 1656.71 cm^{-1} and 1543.41 cm^{-1} for C=C stretching vibration, at 1418.42 cm^{-1}, 1331.42 cm^{-1}, 1268.99 cm^{-1}, 1140.80 cm^{-1} and 1094.16 cm^{-1} for C-O stretching vibration and at 841.16$cm^{-1}$for C-H bending vibration.

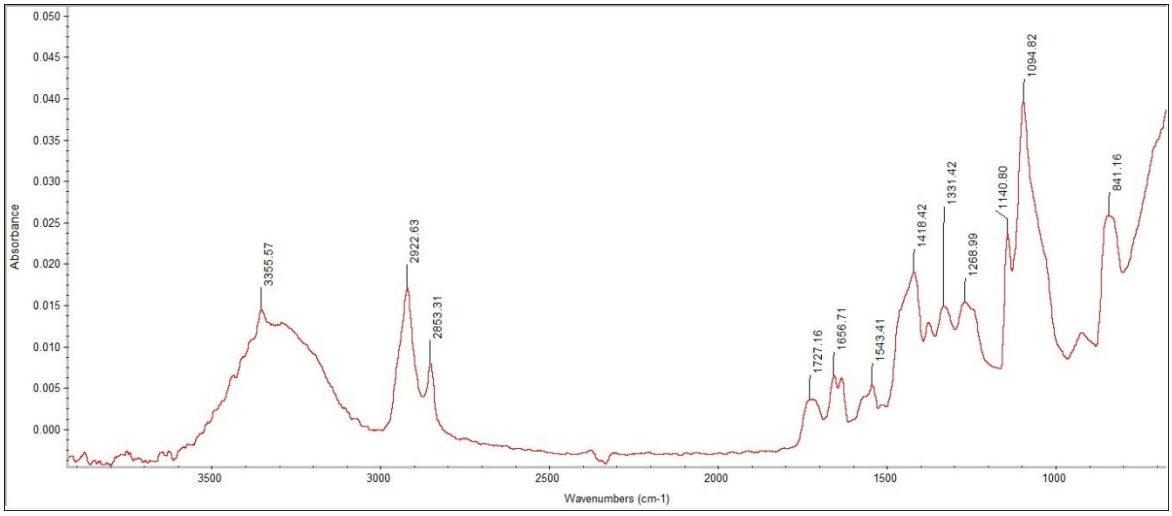

Figure 4.10 The infrared spectra of biocomposite film with 1:1 ratio of PVA to BC content.

From Figure 4.7 to Figure 4.10, it can be seen that the spectrum of the hydroxyl group or OH group were increased with the increased of the ratio of PVA to BC content in the biocomposite film. The present of the hydrogen bonding in the film will give the strong structure to the film with the present of the hydrogen bonding between it. Besides the increasing in the spectrum of the C-H group also can be seen from film with 1:4 to 1:1 ratio of PVA to BC content where this group is strong, broad and multi-banded. More than that, the present of C=O group also give the strong molecular to the biocomposite film. With the combination of bacterial cellulose, polyvinyl alcohol (PVA) and fragrant oil, there was strong interaction between compound and a lot of hydrogen bonds, ionic bonds and a few covalent bonds were presented (Frischen, 2005).

4.5 Scanning Electron Microscopy (SEM)

The microstructures of the films with 1:4, 1:2, 3:4, and 1:1 ratios of PVA to BC content were investigated through SEM, and the results were provided in Figure 4.11 to Figure 4.14. The figures showed the SEM images for the surface of the samples. The 1:4 ratio of PVA to BC content showed roughest morphology and incomplete miscibility between PVA, cellulose and lavender oil. The nanofibrillar structure is clearly can be seen on the surface of the film. Then for 1:2 ratio of PVA to BC, the molecules of nanofibrillar structure on the surface of the film were decreased. The film had smooth morphology compared to the film 1:4 ratio of PVA to BC content. For 3:4 and 1:1 ratios of PVA to BC content, it can be seen on Figure 4.13 and 4.14. The film displayed a relatively smooth morphology and fully homogeneous and compact structure obtained. This is because PVA were completely covered the molecule structure of the blend film where no more nanofibrillar can be seen when 1:1 ratio of PVA to BC content were added to the blend film. By increasing of ratio of PVA to BC content, the morphology of the films will become smoothest but the surface structure would become rougher. Beside the film with highest ratio of PVA to BC content are more compact and stronger compare to the lower ratio of PVA to BC content films.

The addition of some polymers can modify drastically the cellulose biosynthesis (Grande, 2009). This means that when the present of higher percentage of PVA, the biocomposite film would display a relatively smooth morphology and fully homogeneous and compact structure. So, this will avoid the film from easily torn especially after film was immersed or exposed to the water.

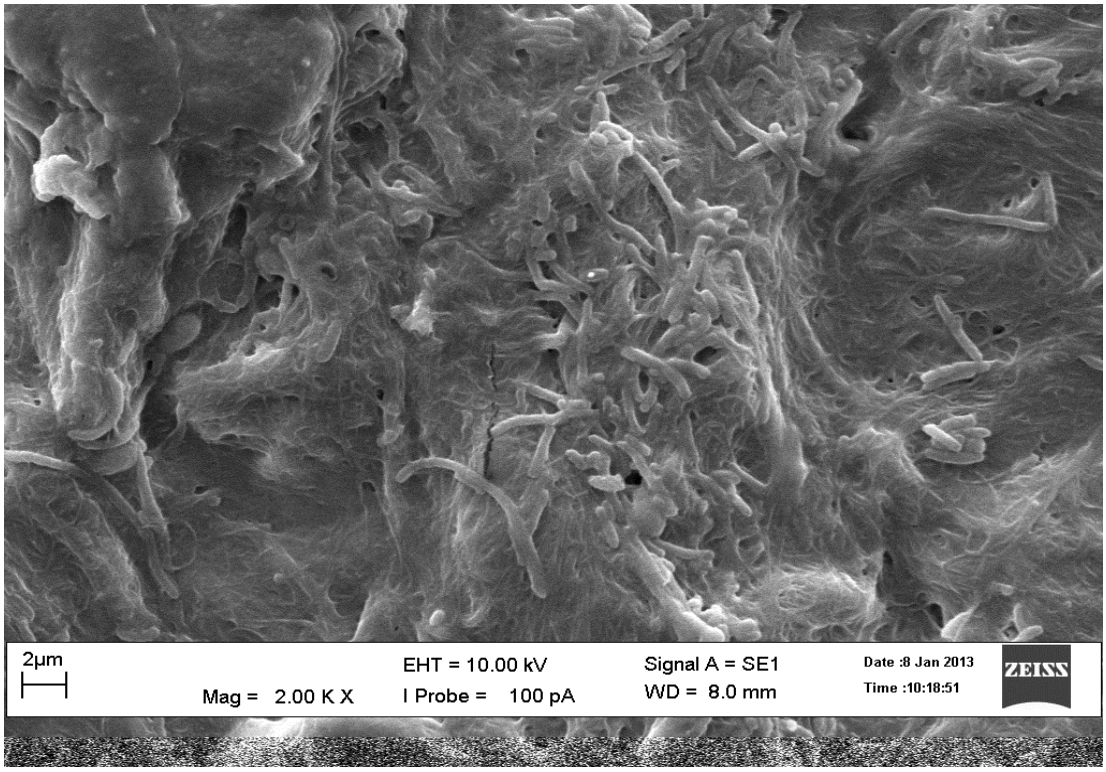

Figure 4.11 SEM micrograph of surface of biocomposit film with 1:4 ratio of PVA to BC content.

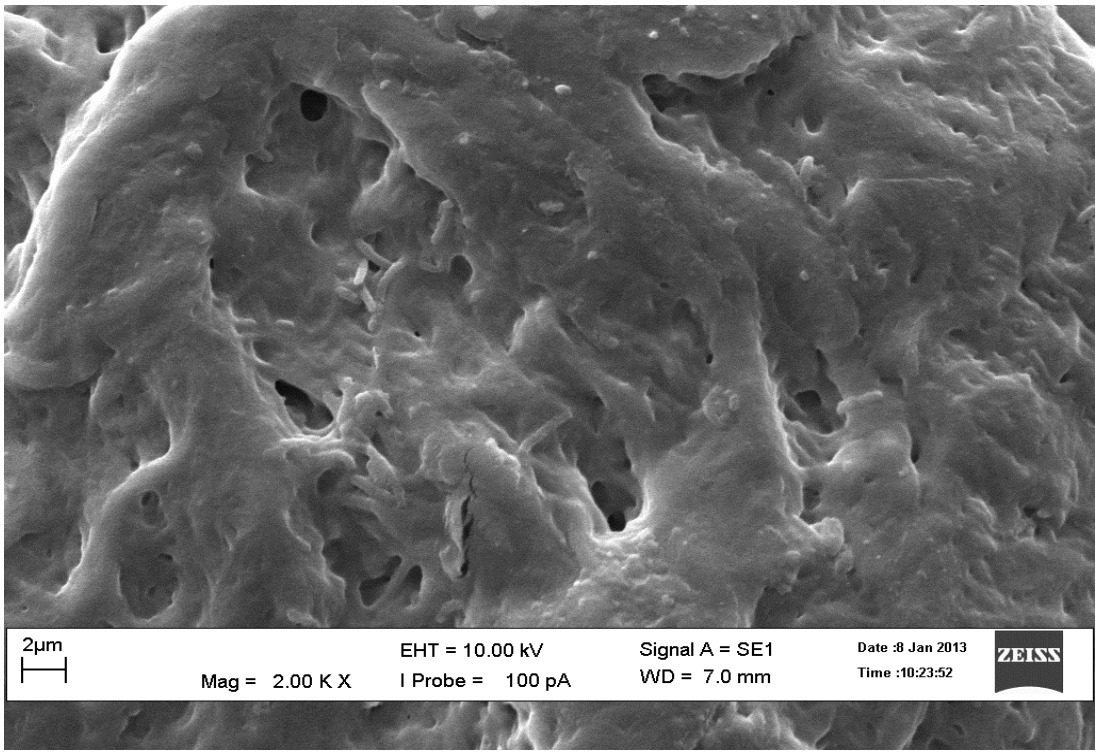

Figure 4.12 SEM micrograph of surface of biocomposit film with 1:2 ratio of PVA to BC content.

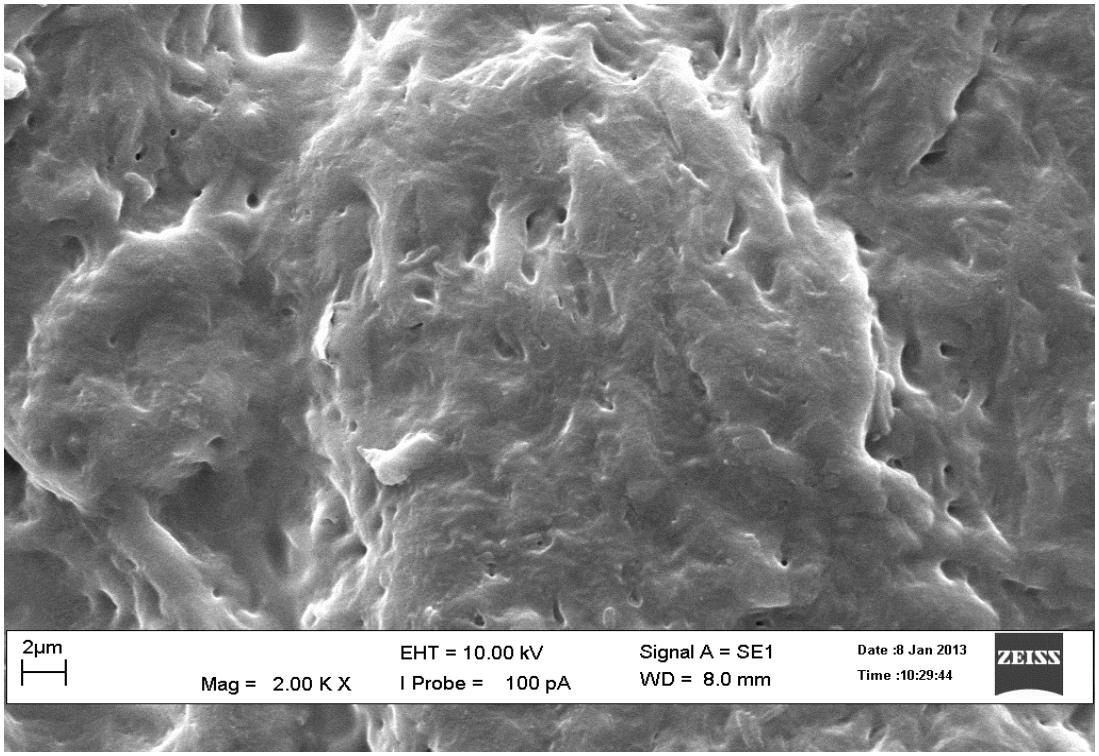

Figure 4.13 SEM micrograph of surface of biocomposit film with 3:4 ratio of PVA to BC content.

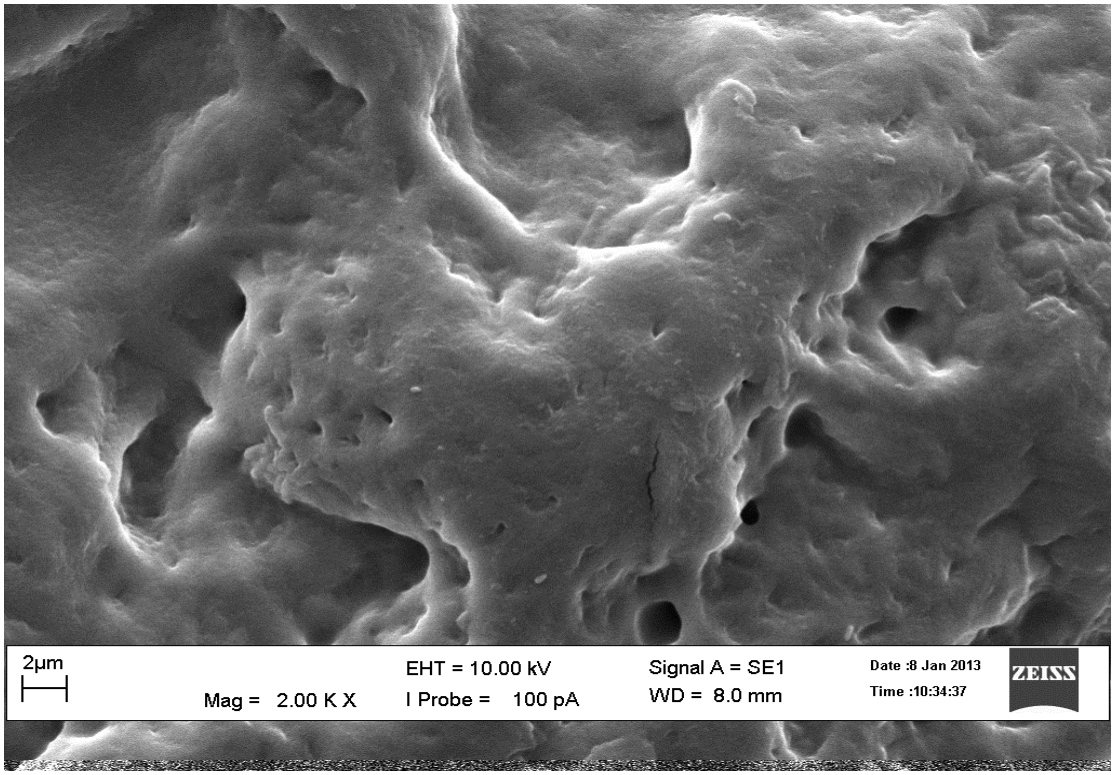

Figure 4.14 SEM micrograph of surface of biocomposit film with 1:1 ratio of PVA to BC content.

4.6 Density

The result from above Table 4.3 and Figure 4.3 showed the value and result of the density measurement by using gas pycnometer and experimental against different ratio of PVA to BC content t in the biocomposite film. From gas pycnometer analysis, the density of 1:4 ratio of PVA to BC content was 363.84kg/m^3, for 1:2 ratio of PVA to BC content was 745.33kg/m^3, for 3:4 ratio of PVA to BC content was 895.28kg/m^3 and for 1:1 ratio of PVA to BC content were 1211.35kg/m^3. Then, for experimental, the density of 1:4 ratio of PVA to BC content was 339.71kg/m^3, for 1:2 ratio of PVA to BC content was 700.98kg/m^3, for 3:4 ratio of PVA to BC content was 850.00kg/m^3 and for 1:1 ratio of PVA to BC content were 1129.74kg/m^3.

Between these two methods an error was occurred because of the medium used during the experiment. For gas pycnometer, the medium used was gas while for the experiment the medium used were liquid. According to the previous study at higher concentration of chitosan the film become more compactness (Krzensiska *et al*., 2007). But the studied was focus on combination of bacterial cellulose and chitosan. But the comparison still can be done because both were polymer and the effect should be not too far for each other.

Beside there was strong interaction between compound and a lot of hydrogen bonds, ionic bonds and a few covalent bonds were presented in the biocomposite film (Frischen, 2005).So, according to him there were many interactions that occurred in the film. So, the higher ratio of PVA to BC content will gave the higher density of film.

Table 4.3 The density value by gas pycnometer and experimental at different ratio of PVA to BC content.

sample	Gas Pycnometer (kg/m^3)	Experimental (kg/m^3)	Percentage error,%
1:1	363.84	339.71	7.10
1:2	745.33	700.98	6.33
3:4	895.28	850.00	5.33
1:1	1211.35	1129.74	7.22

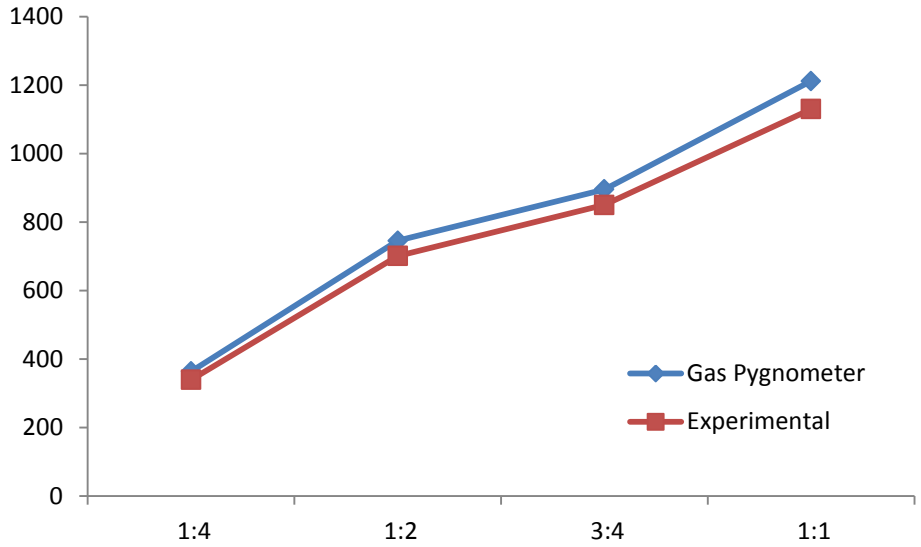

Figure 4.15 The density value by gas Pycnometer and experimental by different ratio of PVA to BC content.

4.7 Water Absorption

Water absorption was important for providing some understanding in about the interaction mechanism between water and film components. From Figure 4.16 the value of diffusion coefficient will be decrease when the ratio of PVA to BC content increase and from Figure 4.17, the maximum moisture absorption was directly proportional to the ratio of PVA to BC content in the biocomposite film. It is because PVA are hydrophilic polymers that are able to retain a considerable amount of water that depends on the structure. There

are also two main sides of PVA for water absorption that is hydroxyl group and also aldehyde group.

According to Klepko and Mel'nechenko (1994), PVA hydrogel contain more water content compared to BC/PVA biocomposite water content. This indicates that PVA are able to retain water batter compared to bacteria cellulose itself. So, the higher ratio of PVA to BC content will give the higher maximum moisture absorption to the film. This was support by Czaja *et al*. (2006), where the biocomposite film must be able to create and maintain a moist environment in the wound.

Table 4.4 The diffusion coefficient and maximum moisture absorption with the different ratio of PVA to BC content in the sample.

sample	Diffusion coefficient	Maximum moisture absorption
1:4	0.39	0.11
1:2	0.27	0.12
3:4	0.19	0.13
1:1	0.12	0.17

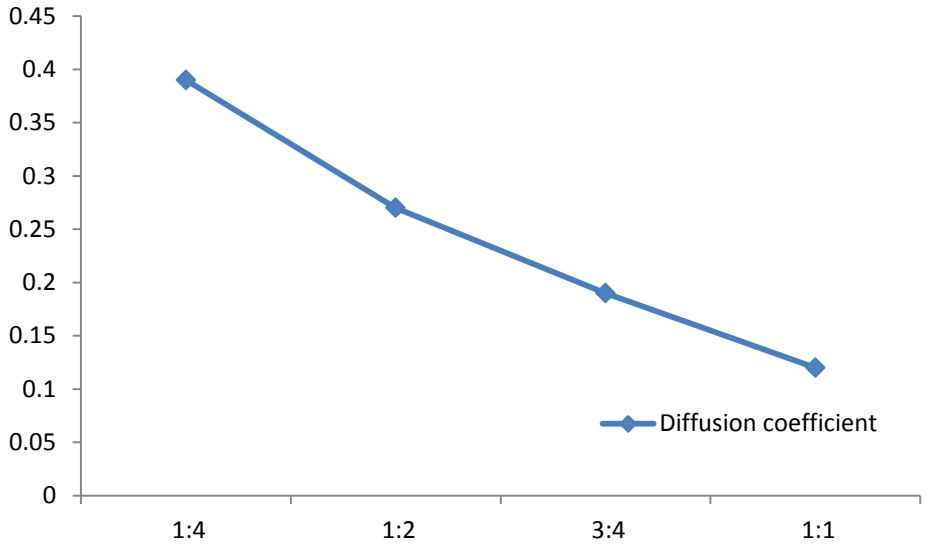

Figure 4.16 Diffusion coefficients against ratio of PVA to BC content in the biocomposite film.

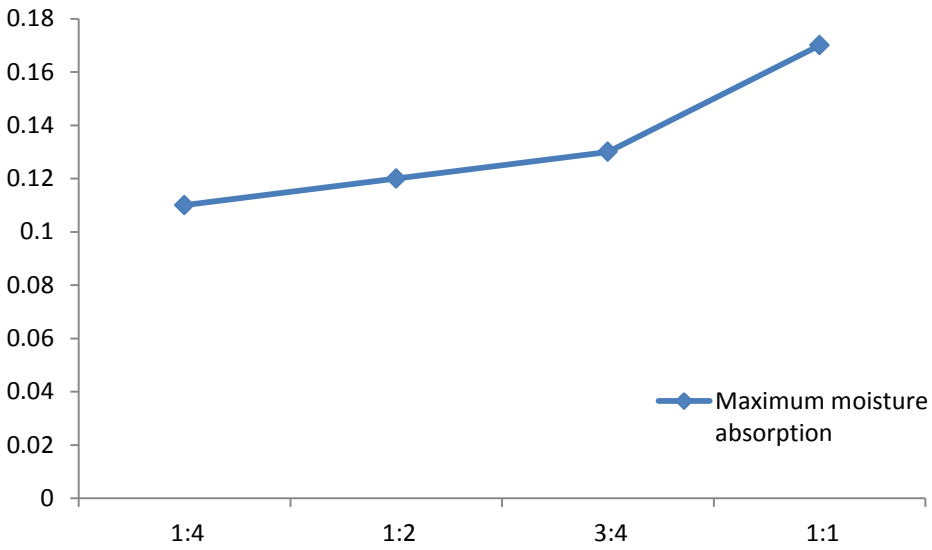

Figure 4.17 Maximum moisture absorbtion against ratio of PVA to BC content in the biocomposite film.

Chapter 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The biocomposite films with different PVA content were synthesized by using mixing and casting process, and the characterization is done by using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), gas pycnometer, antimicrobial test and water absorption to determine the availability of the biocomposite film in wound healing application. To be good in wound healing application, the biocomposite film must be a good wound dressing material where it's must be able to donate moisture, prevent infection, high tensile strength, biocompatibility and can reduce pain (Czaja *et al.*,2006).

The film contains higher ratio of PVA to BC content showed high effectiveness to kill the bacteria and has good water absorption where these were among the good

characteristics to become wound dressing material for wound healing application. Besides, the density of this film was high because of the interaction of OH stretching, C-H stretching and C=O stretching which can increase the strength of the biocomposite film. From the SEM analysis it shows that the structure of biocomposite film with higher ratios of PVA to BC was compacted and was smoothed. Thus this film had a potential to be used as wound dressing material for wound healing application.

5.2 Recommendation

For the recommendation, try to produce the fragrant oil by yourself to make sure the purity of the fragrant used and then use the fragrant oil as the parameter to determine the effect of different ratio of fragrant oil to the film antimicrobial properties. Other than that, the antimicrobial properties of the biocomposite film also test by using either by different type of fragrant oil or combination of several fragrant oil to improve it ability against bacteria. Beside in the next study, other bacteria can be used to determine its effect to this biocomposite film. *S. aureus* are recommended to be use because it is the gram positive bacteria and this study only focus of using *E. coli* where it is a gram negative bacterium. Then we can know the effect of gram positive bacteria to the film.

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APPENDICES

Appendix A Analysis equipment

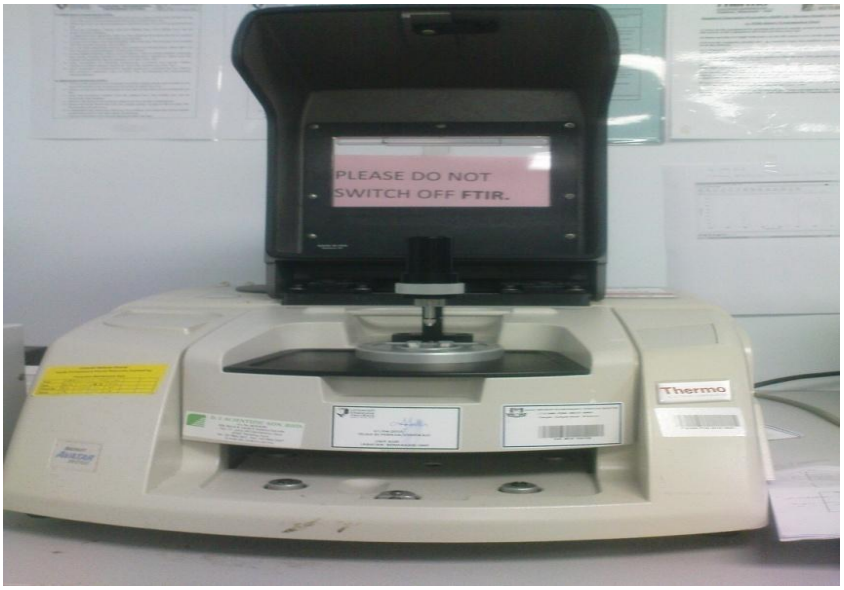

Figure A.1 Fourier Transform Infra-Red

Figure A.2 Gas Pycnometer

Appendix B Preparation of biocomposite film

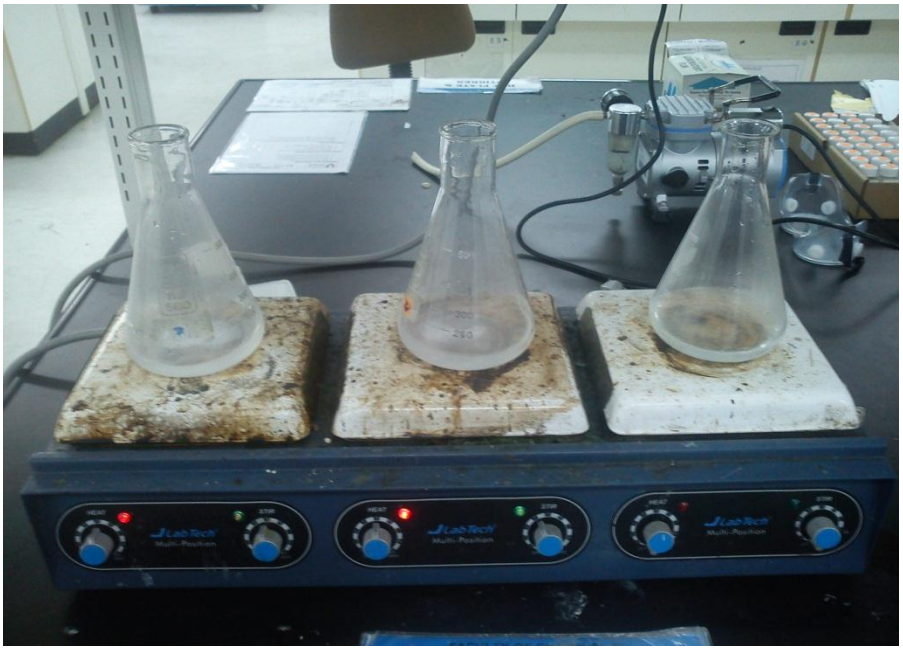

Figure B.1 Heating and stirring of mixture

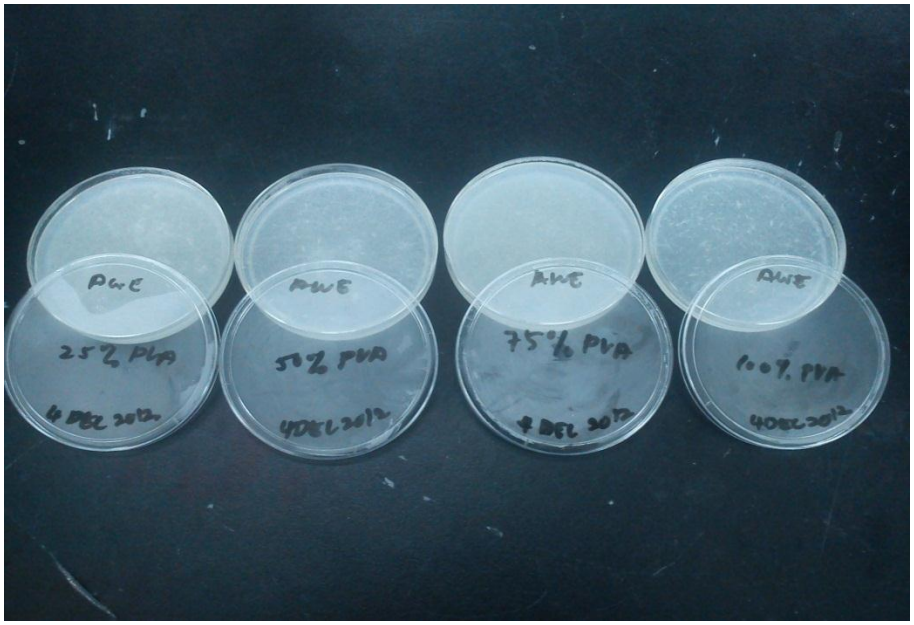

Figure B.2 Biocomposite mixture before drying process**.**

Appendix C Analysis

Figure C.1 Density and water absorption test.