THE FABRICATION OF ANTIBACTERIAL COMPOSITE FROM BACTERIAL CELLULOSE AND BETEL LEAVES

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ABSTRACT

Wound healing was a complicated process that needs to be take care from infection and inflammation. Infection that occurs on wound will slow down the healing process. Therefore the objectives of this research were to produce antibacterial biocomposite from bacterial cellulose with betel leaves. The films were prepared by mixing the bacteria cellulose, betel leaves and gelatin in four different samples that contain from 0% to 70% composition of betel leaves extract. These biocomposite films were characterized by using Universal Testing Machine, Fourier Transform Infrared (FTIR) Spectroscopy, Scanning Electron Microscope (SEM), Gas Pycnometer, Antibacterial Effect Testing, water absorption testing and Biodegradable Testing. The biocomposite film that contains 70% Betel Leaves extract displayed better antibacterial properties, biodegradability and optimum water absorption for wound healing process. The film showed high effectiveness antibacterial activity towards gram positive bacteria rather than gram negative bacteria where the inhibition area was 0.20 cm^2 and the effectiveness activity was 100%. There was about 343.87 mg/L of phenolic content in this sample. The film also showed the highest rate water absorption value which was 1.812×10^{-12} cm^2/s and the film completely degraded in 24 days. The addition of betel leaves extracts and bacteria cellulose can be a promising source to produce biocomposite film that contains antibacterial effect which was suitable for wound healing and also can shorten the wound healing process.

ABSTRAK

Penyembuhan luka adalah satu proses yang rumit yang memerlukan penjagaan yang rapi daripada sebarang jangkitan dan radang. Jangkitan yang berlaku pada luka akan melambatkan proses penyembuhan.Oleh itu, objektif kajian ini adalah untuk menghasilkan antibakteria biokomposit daripada selulosa bakteria dan daun sireh. Biokomposit filem dihasilkan dengan campuran selulosa bakteria, daun sireh dan gelatin dalam 4 sampel yang berbeza dengan mengandungi 0% hingga 70% kandungan perahan daun sireh. Bio-komposit filem ini di kenal pasti dengan menggunakan, *Fourier Transform Infrared (FTIR) Spectroscopy*, Mikroskop Elektron Imbasan, Gas Piknometer, ujian antibakteria, ujian serapan air dan ujian biodegradasi. Biokomposit yang dihasilkan daripada selulosa bakteria dan 70% kandungan perahan daun sireh menunjukkan sifat antibakteria dan biodegradasi yang baik serta mempunyai kadar penyerapan air yang optimum untuk penyembuhan luka. Filem ini menunjukkan tahap keberkesanan antibakteria yang tinggi kepada Gram positif dan Gram negative bakteria dimana kawasan yang terbantut adalah 0.20 cm^2 dan keberkesanan aktiviti antibakteria adalah 100 % dengan kehadiran 343.87 mg/L kandungan fenolik. Filem ini turut menunjukkan kadar serapan air yang tinggi sebanyak 1.812×10^{-12} cm^2/s dan boleh terurai sepenuhnya dalam masa 24 hari. Campuran daun sireh dan selulosa bakteria akan menjanjikan penghasilan bio-komposit yang mempunyai kesan antibakteria serta sesuai bagi merawat luka dalam masa yang singkat.

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ ABBREVIATIONS

^{0}	- Degree
%	- Percent
C	- Celcius
g	- gram
cm	- centimeter
Kg	- Kilogram
L	- Liter
mL	- mililiter
min	- minute
s	- second
BC	- Bacterial Cellulose
BL	- Betel Leaves
EAA	- Effectiveness Antibacterial Activity
CPU	- Number of colony forming unit

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

A wound is a break of the outer layer of skin which is epidermis and can be caused due to various reasons, but the most ordinary ones are scrapes, burn, lacerations, punctures and cuts. All the tissues heal after a certain period of time and leaving scars. Wound healing is a process of repairing the injury on the skin and other soft tissues. It is complex and fragile process, which can be complicated by infection and inflammation. This process requires the involvement of many different tissue, cell type and matrix component. Wound can be classified by 3 stages which are wounds are characterized by redness and swelling, wounds partially penetrate the skin and wounds involve damage to muscle or bones.

All wound are heal in inflammatory stage, proliferate stage and maturation and remodeling stage. Poor healing wounds may slow down the healing process due to the infection of a wound with a large number of bacteria .Signs of infection include red skin

around the wound, swelling, warmth and foul odor. Anaerobic bacteria such as *bacteroides*, *clostridium* and *streptococcus* may be active at deeper levels of the dermis, insulated from the healing influence of oxygen (Sobotka at. el., 2010).

Thus, it is important that wounds be treated early so that an infection does not further develop. A wound needs a proper care to prevent infection or scars. The healing process is very important and it becomes essential to take good care of any injured area. Cleanliness of the wound site must be maintain throughout the healing process.

There are a few herbs that can be used as a home remedy for wound infection. Betel leaves, locally known as Sireh is a well known plant in Malaysia traditional medicine. The leaves have a spicy taste and yield essential oil that are widely use for the antifungal and antiseptic. It is also contain rich Carotenes, Ascorbic Acid and Phenolics. The content of phenol in the antiseptic properties is five times more effective than with ordinary phenol. In the leaves itself, it contained eugenol which is able to prevent premature ejaculation, eradicate the fungus *Candida albicans*, and pain relief. These plants also use to overcome the body and mouth odor, mouth sores, nosebleeds and sores.

Bacterial cellulose (BC) is pure a cellulose that produced by bacteria such as *Acetobacter Xylinum* which suite for medical application such as artificial blood vessel artificial skin and wound dressing (Jagannathi at el., 2010). Only a few bacterial species produce extracellular Cellulose in form of fiber.

In the new era with advanced technology, there should have the modern wound care dressing material. It will provide easy wound coverage, display high mechanical strength, elasticity, conformability and biocompatible. The designing a film from bacteria cellulose will become a good dressing material for wound healing with few of researches.

1.2 Problem Statement

Nowadays, the procedure for wound healing is very complicated. Leaving a wound uncovered will cause worse effect where wound area will get dirty or be irritated by clothing. The area of the wound should be cover with an adhesive strip or with sterile gauze and adhesive tape. In order to make the wound clean and dry, the bandage should be change every day. At the same time antibiotic ointments should be uses to help healing by keeping out infection and keeps the wound clean and moist. Given these problems, development of an alternative technique or dressing to reduce wound infection is of importance.

This research will concentrate on designing the film from bacterial cellulose with betel leaves. On the basis of its traditional use, this plant was selected for antibacterial activities in wound healing effect. In fact, these leaves were available abundantly in the garden or backyard of the numerous houses and the function was most likely for wound healing. The combination between bacteria cellulose and this herb plant will become a simple step for wound healing that contain antibacterial effect.

1.3 **Research objective**

The objective that needs to be achieved in this research was to produce antibacterial biocomposite from bacterial cellulose and betel leaves

1.4 Scopes of the study

The scopes of this research are:

1. To produce biocomposite from bacteria cellulose and betel leaves
2. To analysis the composition of betel leaves from 0% to 50% in order to gets the ideal biocomposite film for wound healing.
3. To characterize the biocomposite film by using Universal Testing Machine Fourier transform infrared spectroscopy (FTIR) , Scanning Electron Microscopy (SEM) and Gas Pycnometer
4. To test the antibacterial activity, water absorption and biodegradability of biocomposite film

1.5 Significant of the study

The designing biocomposite film from bacteria cellulose and betel leaves can be potential to become modern wound care dressing material which are non toxic, not irritate the patients skin and transparency which allows for continuous clinical observation of healing process. The film was able to provide barrier against infection because of the special characteristic of betel leaves that contain the phenol which has a powerful antiseptic properties. Furthermore, the film is more convenient and cost less than drugs prescription.

CHAPTER 2

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LITERATURE REVIEW

2.1 Bacteria Cellulose

Bacterial cellulose (BC) is a linear polysaccharide which have the molecular formula $(C_6H_{10}O_5)n$ same as plant cellulose, but their physical and chemical features are different (Saibuatong and Phisalapong, 2010). The cellulose is produced by various species of bacteria such as those of genera Gluconacetobacter formerly Acetobacter, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Rhizobium, Sarcina and Salmonella (David et al., 2008). It is also is one of natural, biodegradable and edible polimer. Besides that bacterial cellulose have unique properties such as high mechanical strength, high crystallinity and high pure nanofibrillar network structure.

The bacterial cellulose has established for various applications such as electronic paper, optically transparent composites, reinforcing agent for paper and many more. Due to its biocompatibility, bacterial cellulose has been investigated in the biomedical field as tissue engineering and in medical application such as wound dressings and artificial blood vessel.

Bacterial cellulose that produced by *Acetobacter Xylinum* in static cultures is initially extruded from the cell surface. These bacterial cellulose has been produced in a thin film on the surface of fermentation broth. Glucose and fructose as the carbon source and acetic acid as the energy source are combined with a precise control of pH and dissolved oxygen levels will highly improved cellulose yields. The film grows downward since cells that are entrapped into the film become inactive or die from lack of oxygen (Cheng et al., 2009).

2.2 Acetobacter Xylinum

Acetobacter Xylinum is a rod shaped, aerobic and gram negative bacterium which has ability to synthesis high quality cellulose organized as twisting ribbon or microfibrillar bundle (Setyawaty al et., 2009). It also reclassified as Gluconacetobacter Xylinus that will grow at the optimum temperature, 25-30 ^{O}C and the optimum pH range from 5.6 to 6.2.

The *Acetobacter Xylinum* bacterium has been used as a model system for investigation of biosynthesis of cellulose due to its ease of handling and it ability to produce cellulose as an extracellular product. The cellulose has produced at the surface of medium from organic substrates like coconut water and tomato juice. The cellulose is a thin slimy and transparent layer which forming after 10-15 days (Jagannathi at el., 2010).

This bacterial cellulose has become a new industrial material because of it properties which is high crystalline, has high water absorption capacity and good mechanical properties (Saibuatong and Phisalapong, 2010). Owing to its unique properties, the cellulose that produced from *Acetobacter Xylinum* has a good potential to transfer antibiotics or other medicine into the wound and at the same time become an efficient physical barrier against any external infection.

2.3 Betel Leaves

The betel leaves is widely grown in the South East Asia. It is a spice whose leaves have medical properties. The plant is evergreen and perennial with glossy hearth-shaped leaves and grows to a height of about 1 meter. The plant also prefers warm and humid conditions to grow.

Betel Leaves consists of 85.4 percent of moisture, 3.1 percent of protein, 0.8 percent of fat, 2.3 percent of mineral, 2.3 percent of fiber and 6.1 percent of carbohydrate per 100 gram of Betel Leaves (Hayati et al., 2009). The mineral contents of betel leaves are Calcium, Carotene, Thiamine and Vitamin C.

Besides that, betel leaves also contain essential oil which a light yellow liquid of aromatic odor and sharp burning in taste. This essential oil contain a phenol which has powerful antiseptic properties (Mahmood et al., 2005) .

The medical application of betel leaves has been well known for a long time. It leaves have healing power for wound, headaches, respiratory disorders and sore throat (Prakash at el., 2010). The leaves with a strong pungent an aromatic flavor are widely consumed as mouth freshener.

2.4 Biocomposite

Biocomposite are materials that consist of two or more distinct constituent to obtain complex chemical, mechanical and biological properties. It also known as reinforced material. One or more of the biocomposite phase are derived from biological origins like plant fibers from crops such as cotton, recycled wood, waste paper or regenerated cellulose fiber (Arizaga at el., 2009). Biocomposites are also biodegradable material. They are broken down into CO_2 and water by microorganisms. Some of these biodegradable biocomposite are compostable. In addition, biocomposites have the

potential to cut carbon emissions and reduce CO_2 quantities in the atmosphere. This is because the CO2 released during degradation can be reabsorbed by crops grown to replace them, making them close to carbon neutral. The majority of biocomposite are used in the automotive, construction, furniture and packaging industries.

There was reported that most of the synthetics biodegradable composites contain hydrolysable linkages along the polymer chain. Natural macromolecules likes protein, cellulose, and starch generally degrade in biological system by hydrolysis followed by oxidation. The hydrophilic and hydrophobic character greatly affects the biodegradation of the composites. A composites containing both hydrophobic and hydrophilic segment seems to have higher biodegradability than the composites that containing either hydrophobic or hydrophilic structure only (Lucia et al 2008). The biological environment, in which composites are present, includes the biological agent responsible for the deterioration of composite substance. Biological agent such as bacteria, fungi and enzyme consume a substance as food sources so that the original form is disappears.

The fungi are the important microorganisms in causing the degradation of material. Fungi are nucleated, spore-forming, which reproduce both sexually and asexually. Most of them possess filamentous, somatic structures, and cell walls of chitin or cellulose. Fungi are producing the enzyme which breaks down nonliving substrate in order to supply nutrient material present in composite composition. Certain environmental conditions are essential for optimum growth and degradation activity. Bacteria can be single cell rods, cocci or spiralla. The degradative action of bacteria is chiefly a result of enzyme production and resultant break down of the nonliving substrate in order to obtain nutrient materials. Bacteria present in soil are important agents for material degradation.

2.5 Wound

Wound is specifically refers to a sharp injury which damages the dermis of the skin. The skin has the outer epidermis, dermis and fatty subcutaneous layer. Wounds can penetrate any of these layers and skin infections can spread into them. Besides that wounds are refer as the superficial cuts, scrapes or scratches and also include punctures, burns or may be the result of surgical or dental procedures. The microorganisms that are infect into the wound is depend on the wound's extent and depth, the environment in which the wound occurs, and the microorganisms present on the person's skin (Carlson at el., 2008).

A wound infection is the presence of replicating microorganisms within a wound or surrounding tissue that can cause host injury or a systemic toxicity. The infection are causes inflammation, tissues damage and slow healing process (Farstvedt et al., 2004). Bacteria and fungi can cause the wound infection. Bacteria are divide to aerobic, anaerobic and microaerophilic and some of them can cause the infection such as *Staphylococcus aureus, E. coli, Pseudomonas, Klebsiella* and *Acinetobacter* (Mellisa et al., 2004). The symptoms of wound infection include redness, swelling, warmth and tenderness. Sometimes the infection may cause scaling, pain or itching.

The risk of wound infection can be minimized with prompt and proper wound cleansing and treatment. Numerous typical wound mendication are available today likes wound cleanser and antiseptic. These products should be used in the initial phases of previous wound management to decrease bacterial load and rid the previous wound of necrotic tissue. The antiseptics are used in the healing process to reduce bacterial numbers and subsequently to reduce the chances of infection. Antiseptic solutions are have some toxic effects that may do more harm than good.

Besides that, a large number of herbal therapies and combinations of therapy presently exist for wound care. Herbal preparations are only one component of

alternative medicine, which encompasses a wide variety of approaches. Aloe Vera is reported to stimulate wound healing (Ammayappan et al., 2009). It has antibacterial, antifungal and stimulates collagen production. Honey also has many potentially useful properties for wound healing including a broad spectrum antimicrobial activity, inflammatory actions and stimulation of new tissues growth.

2.6 Fourier Transform Infrared Spectroscopy (FTIR)

The Fourier Transform Infrared is the prefer method of infrared spectroscopy. It is the analysis technique that provides information about the chemical bonding or molecular analysis technique structure of materials, whether organic or inorganic (Lucia et al., 2008). This technique offers a non destructive alternative to chemical measurement technique for qualitative characterization. This tool also has the ability to readily carry out the multi component analysis.

The infrared radiation will passed through a sample. A molecule that is exposed to infrared ray absorbs infrared energy at certain frequencies. The resulting spectrums represent the molecular absorption and transmition. Fourier Transform Infrared Spectroscopy (FTIR) can easily manipulate spectral information by using computers. Its advance software is equipped to handle the calibration.

In the previous research analysis, the pure bacterial cellulose shows characteristic peaks between 984 and 1106 cm^{-1} corresponding to the C–O bond stretching. The band at 1650–1578 cm^{-1} is assigned to C═O stretching and the bands present between 3200 and 3400 cm^{-1} correspond to O–H stretching modes in alcoholic groups (Phisalapong et al,. 2009). Besides that, the previous research also shows that the concentration of betel leaves will affect the peak intensity of C=O and C-O stretching because of the presence of hydroxyl group in phenolic contents (Hayati et al., 2009).

2.7 Scanning Electron Microscopy (SEM)

The Scanning Electron Microscopy is an instrument that reveals the sample's information including chemical compositions, crystalline structure and crystalline orientation. Scanning Electron Microscopy also has the combination of higher magnification, larger depth of field and greater resolution that make it as the most heavily used instruments in research areas. This instrument is the combination between a few essential components such as electron source, electron lenses and detectors for all signals of interest.

The high energy electrons is strike the sample and the variety of signal are generated. The three signals which provide the greatest amount of information in SEM which are the secondary electrons, backscattered electrons, and X-rays. The signal of secondary electrons is commonly used for imaging samples, while the backscattered electrons signal will determine the crystal structure and orientations of mineral and also for imaging sample. Detectors are collected these X-rays, backscattered electrons, and secondary electrons and convert them into a signal that is sent to a screen for the final images.

The morphology of bacterial cellulose composite is a very important parameter because it is closely related with their mechanical performances. The Scanning Electron Microscopy images of all bacterial cellulose composites provide evidence of the strong interfacial adhesion between two component as shows by the complete impregnation of the bacterial cellulose nanofibrillar network into the polymeric matrices (Jatupaiboon et al., 2008).

2.8 Gas Pycnometer

A common laboratory device for measuring absolute density of a solid is a gas pycnometer. The absolute density or also termed the true density is obtained when the volume measured excluded the pores. The solid density is expressed as ratio of the total mass of solid material to the volume of the material. It is expressed in kg/m3. Typically, the solid phase density is measured with a helium pycnometer. The Helium pycnometer is much more accurate because it easily, quickly and thoroughly fills the minutest pore spaces. The helium is selected because it molecules are smallest of all existing molecule and can enter practically all pores.

The solid and closed cell volume of the sample is calculated from the relationship of the calibrated cell volumes and the pressure before and after expansion. The closed cell percentage is calculated from the solid volume and the measured geometric volume of cylindrical sample. The density of material depends on the molecular packing. For solids, the density is vary with the crystal structure and degree of crystallinity. The density also depend the history of preparation and treatment of the material.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter present the methodology used to produce the antibacterial biocomposite from bacterial cellulose and betel leaves. In the first step, the culture bacteria (*Acetobacter xylinum*) were culture in the culture medium to get bacterial cellulose. Next, the biocomposite was fabricated by blending bacterial cellulose and gelatin with different percentage of betel leaves extract. Finally, the films were characterized by antibacterial testing, phenolic content analysis, Fourier Transform Infrared Spectroscopy (FTIR) analysis, Scanning Electron Microscopy (SEM), Water absorption testing, biodegradable testing and density analysis.

3.2 Material

The stock culture of *Acetobacter xylinum* was supplied by Malaysia Agricultural Research and Development Institute, Serdang Selangor and the fresh betel leaves were collected from Felda Chini 3, Pekan Pahang. The reagent, Ammonium Sulfate ($NHSO_4$), Sodium Hydroxide (NaOH), Glycerol, Acetic Acid, ethanol and Folin Ciocalteu Reagent were purchased from Sigma, Chemical Co. St. Louis.

3.3 Experimental Procedure

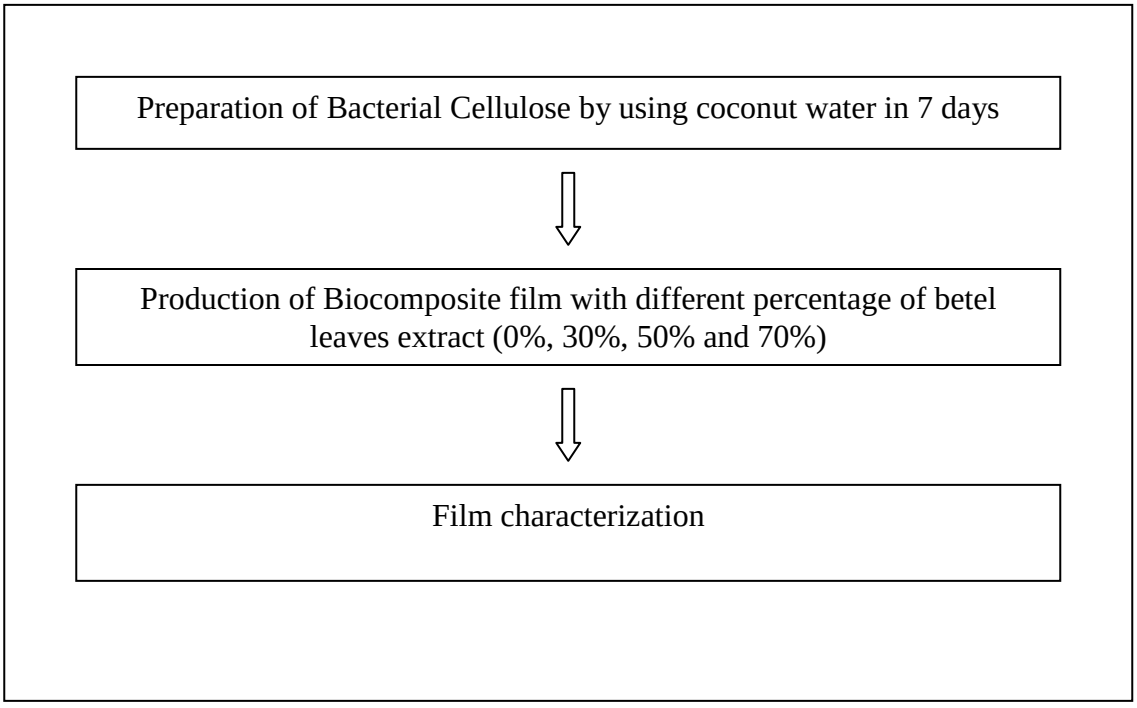

Figure 3.1: Experimental procedures for fabrication of antibacterial composite film

In Figure 3.1, the procedures shown were the overall procedure done to fabricate film sample. In the first step, the stock culture was inoculated into medium culture for 7 days. The medium culture was prepared by using coconut water, sucrose, ammonium sulfate and acetic acid. In the next step, the film samples were produced. Bacterial cellulose was dissolved in gelatin and various percentage of betel leaves extract. The last step was film characterization. All the films sample were characterized by using universal testing machine and the series of testing for antibacterial activity, water absorption and biodegradability.

3.3.1 Preparation of Bacteria Cellulose

The culture medium for the inoculums was coconut-water containing 8.0% sucrose, 0.5% ammonium sulfate $(NH_4)_2SO_4$ and 1.0% acetic acid. The medium was sterilized at 121 °C for 20 min. The 100 mL of a stock culture was then inoculated into 1000 mL of medium and incubated at 30 °C for 7 days in static culture. After 7 days a white membrane had formed at the medium interface. The membrane was purified by washing with deionized (DI) water and then treated with 1% (w/v) sodium hydroxide solution at room temperature for 24 hour to remove bacterial cell followed by a rinse with DI water until pH become neutral (Jatupaiboon et al., 2008).

3.3.2 Preparation of Betel Leaves Extracts

The fresh betel leaves were washed with distills water and air dried for 3-5 days in the shade and cutted into small pieces. The 500 g of betel leaves were soaked with 80% (v/v) ethanol and kept for 48 hour at room temperature (Nalina et al., 2010). The aqueous extract of Betel Leaves obtained was filtered using filter paper. After extraction, the extract was collected and the co-solvent, ethanol was removed with vacuum rotary evaporator under reduce pressure at 78 °C for 3 hour.

3.3.3 Preparation of Biocomposite Film

The 7 % w/v bacteria cellulose was dissolved in 2.5% v/v glycerol with the various percentages of betel leaves extract (0%, 30%, 50% and 70%) under magnetic stirring for 1 h at room temperature. Than, the solutions were poured into petri dish and dried at 60 °C for 4 h (Phisalaphong et al., 2010) . The obtained series of film were neutralized by 1M of Sodium Hydroxide (NaOH) solution for 2 hours and washed with distilled water. Finally, the wet films was wiped with a filter paper to remove the excess water present on the surface of the membrane and allowed to dry at room temperature for 24 h.

3.3.4 Film Characterization

3.3.4.1 Antibacterial Properties

3.3.4.1.1. Disc Diffusion assay

The 20 mL nutrient agar plate were inverted and dried at 37 ^{0}C for 30 minute to obtain solid nutrient agar plate. The pieces of film sample with diameter 8 mm was put onto nutrient agar. The culture of bacteria was spread over the surface of the agar plate using a sterile glass spreader. The bacteria used in this testing were *Escherichia coli* (E. coli) and *Staphylococcus aureus* (S. aureus). The agar plates were incubated overnight at 37 ^{0}C. The diameters of the inhibition zones, which appeared on the surface, were measure in five directions. Average values were used for calculation of the inhibition zone area (Altiok et al., 2010).