CHAPTER IV

EXPERIMENTAL

4.1 Materials

4.1.1 Microbial Strains

The *A. xylinum* strain AGR60 was isolated from *Nata de coco*. The stock culture was kindly supplied by Pramote Thammarad, the Institute of Research and Development of Food Product, Kasetsart University, Bangkok, Thailand.

4.1.2 Chemicals

The details of chemicals used in this experiment are shown in Table 4.1

Table 4.1 The chemicals used in this experiment

Chemical	Supplier
Sucrose	Ajax Finechem
Ammonium sulfate	Carlo Erba
Sodium hydroxides	Carlo Erba
Acetic acid	BDH
Gelatin type A (Bloom 300)	Sigma Chemical
Tannic acid	Fluka

4.1.3 Equipments.

-Scanning electron microscopy, SEM (JOEL JSM-5410LV, Tokyo, Japan)

-Fourier Transform Infrared (FT-IR) spectrometer (Perkin Elmer Spectrum One Massachusetts, USA)

-Instron testing machine (Instron 5567, NY, USA)

-X-ray diffraction (Bruker AXS Model D8 Discover,USA)

-Oxygen permeation analyzer (Illinois Instruments, Model 8000, Johnsburg, IL)

-Autoclave (Model Tomy Autoclave SS-325, Nerima-ku, Tokyo, Japan)

-Vacuum Oven (WTB Binder Model VD 23, USA)

4.2 Culture Media and Method

4.2.1 Preparation of BC-gelatin film from biosynthesis by *A. xylinum*

Cell cultivation was performed in a simple and inexpensive way following the procedure developed by Pramote Thammarad (The institute of Food Research and Product Development, Kasetsart University). The medium 100 ml for the inoculums was coconut-water with 5g of sucrose, 0.5g of ammonium sulfate and 1 ml of 30.0%v/v acetic acid. The medium was sterilized at 110 °C for 5 min. The 5% v/v precultures were prepared by transferring 10 ml of a stock culture to 200 ml and incubated statically at 30 °C for 7 days. The experiment was designed to test effects of different gelatin concentrations supplemented in culture medium with 0,1,3,5,7,10% w/v. The preculture broth was added to the main culture in medium which supplemented with different gelatin concentration. The 75 ml activated

medium was inoculated in a 14.5 cm diameter of Petri-dish and kept at 30 °C for 7 days.

All sample films, which were hereafter called "Biosyn-BCG", were first purified by washing with DI water for 1 h and then was treated with 1% w/v NaOH at room temperature for 24 hours to remove bacterial cells followed by a rinse with DI water until pH came to 7. Afterward, the BC-gelatin film was vacuum-dried at temperature of 40 °C for 2 hours. Then, air-dried at room temperature (30 °C) for 2 days and stored in plastic film at room temperature.

4.2.2 Preparation of BC-gelatin by impregnation of BC gels with gelatin solution

The purified BC hydrogel was immersed in an aqueous solution of gelatin with concentration of 0, 15 and 30% w/w at 50 °C for 1 week and then immersed in 3% w/v tannic acid aqueous solution for 2 days at room temperature to make a chemical cross-linking reaction (Heijimen *et al.*, 1997; Yasuda *et al.*, 2005).

All sample films, which were hereafter called "Impreg-BCG", were washed several times by DI water for removing residual tannic acid with DI water. Afterward, the BC-gelatin film was vacuum-dried at temperature of 40 °C for 2 hours. Then, air-dried at room temperature (30 °C) for 2 days and stored in plastic film at room temperature.

4.3 Characterizations of BC-Gelatin Film

The BC-gelatin films were characterized by Scanning electron micrographs (SEM) for investigating morphology, by Instron testing machine for determining tensile properties, by Fourier transform infrared (FT-IR) spectrometer for identifying the chemical structure, by X-ray diffraction(XRD) for finding crystallinity index, Oxygen permeation tester for measuring oxygen transmission rate (OTR), Water vapor permeation tester for measuring water vapor transmission rate (WVTR), Water absorption capacity, Antibacterial ability, Antifungal ability and also *in vitro* study of cytotoxic activity of developed films against Vero cells in individual wells of twenty-four-well plates.

4.3.1 Scanning Electron Microscope (SEM)

The examination of the surface properties was performed by JOEL JSM-5410LV scanning electron microscopy (SEM) (Tokyo, Japan) at Scientific and technological research equipment centre, Chulalongkorn University. The BCG films in reswollen form were dehydrated in ascending grades of ethanol and dried in a Tousimis Samdri-780 critical point dryer (Maryland, USA) using liquid carbon dioxide as transitional fluid. The BCG dried films were sputtered with gold in a Balzers-SCD 040 sputter coater (Balzers, Liechtenstein). The accelerating voltage was adjusted to 15 kV. The specimens were examined at magnification 10,000X for surface morphology and both 10,000X and 100X for cross sectional morphology.

4.3.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is used primarily to identify the chemical structure of the sample. FTIR spectra of the developed film were recorded with Perkin Elmer (Spectrum One, Massachusetts, USA) in the region of 4000–450 cm^{-1} at Scientific and technological research equipment centre, Chulalongkorn University.

4.3.3 Mechanical Testing

The tensile strength and elongation at break of both dried and reswollen BCG film was measured by Instron Testing Machine (Instron 5567, NY, USA) at Polymer Engineering Laboratory, Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University. The load cell capacity was 1kN. The film samples were cut into strip-shaped specimens 10 mm width and 10 cm length. The test conditions followed ASTM D882 as a standard test method for tensile elastic properties. Two ends of the specimens were placed between the upper and lower jaws of the instrument, leaving a length of 6 mm of film in between the two jaws. Extension speed of the instrument was 10 mm/min. The tensile strength and break strain were the average value determined from at least five specimens.

4.3.4 X-Ray Diffraction

X-ray diffraction patterns of the BCG films were determined with a diffractometer (Bruker AXS Model D8 Discover, USA) at Scientific and technological research equipment centre, Chulalongkorn University. The operation conditions were as follows: Cu Target, 40 kV Voltage, 40 mA Current, 5-40 degree angle, 0.02 degree increment and scan speed of 0.2 sec/step with VÅNTEC-1 Detector (super speed detector). The crystallinity index (*C.I.*) was calculated from the reflected intensity data using the Segal *et al.* method, and calculated using the following formula:

$$C.I. = \frac{I_{020} - I_{am}}{I_{020}}$$

Where I_{020} is the maximum intensity of the lattice diffraction, and I_{am} was the intensity of amorphous part of the sample at $2\theta = 18^{\circ}$.

4.3.5 Water Absorption Capacity (WAC)

Water absorption capacity (WAC) was determined by immersing the preweighted of dried BCG film in distilled water at room temperature until equilibration. The film was then removed from the water. After excess water at the surface of the film was blotted out with Kim wipes paper, the weight of the swollen film was measured and the procedure was repeated until there was no further weight change. Water content was calculated using the following formula:

$$WAC(\%) = \frac{W_h - W_d}{W_d} x100$$

Where W_h and W_d denoted the weight of hydrate and dry membrane, respectively.

4.3.6 The Oxygen Permeability Measurement

Oxygen transmission rate (OTR) of the dried BCG films was determined with a oxygen permeation analyzer: Illinois Instruments (Johnsburg, IL) Model 8000 at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test condition followed ASTM D3985. The determination of OTR was done at 23°C and 0% relative humidity. The film was held in such a manner that it separated two side of test chamber. One side was exposed to a nitrogen atmosphere. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

4.3.7 The Water Vapor Permeability Measurement

Water vapor transmission rate (WVTR) of the BCG film with area of 50.00 cm^2, was measured at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test conditions followed ASTM E-96 with desiccant method. The determination of WVTR was done at 38 °C and 98% relative humidity. The test specimen was sealed to the open mount of test dish containing a desiccant, and the assembly placed in a controlled atmosphere. Periodic weighting was performed to determine the rate of water vapor movement through the specimen into the desiccant.

4.3.8 Antibacterial Test

The antibacterial test of cross-linked Impreg-BCG30 and uncross-linked Impreg-BCG30 films against *Escherichia coli* Gram (-) and *Staphylococcus aureus* Gram (+) bacteria was determined at Microbiology Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University. The film samples were cut into 25 mm width and 50 mm length. Testing of antibacterial activity of the films was performed according to the method described by AATCC TM 147-1998 (Anti-bacterial Activity Assessment of Textile Materials: Parallel Streak Method). The samples used for the antibacterial assay were sterilized by using UV irradiation for 20 min in each side. The incubation was 24 hours at 37 °C.

4.3.9 Antifungal Test

Testing antifungal activity of the cross-linked Impreg-BCG30 and uncross-linked Impreg-BCG30 films was performed according AATCC 39-1989(Assessment on Textile Materials: Mildew and Rot Resistance of Textile) at Microbiology Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University. The film samples were punch into round-shaped sample of 3.8 cm diameter. The samples used for the antibacterial assay were sterilized by using UV irradiation for 20 min in each side. The test was performed in the AGAR plate with *Aspergillus niger* for a week of inoculation at 30 °C.

4.4 Cell Study

Cytotoxicity evaluation of BCG films against Vero cells, which were isolated from monkey kidney cell were kindly evaluated by Pongpun Siripong and Jantana Yahuafai at Natural Products Research Section, Research Division, National Cancer Institute of Thailand. The dried BCG films were punched into round-shaped samples of 16 mm diameter. The samples were sterilized by autoclaving at 121 °C for 10 min and transferred aseptically to 24-well culture plates. The experiments were conducted in triplicate. One milliliter of DMEM culture medium with 10% Fetal calf serum was added to each well to equilibrate the samples for 30 min and then removed. The metallic ring and 0.5 milliliter of DMEM culture medium with 10% Fetal calf serum was added to each well before cell seeding. Briefly, Vero cells were seeded into 24-well culture plates at an initial density of 6×10^4 cells per well on the BCG film and the control. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 16 h. Then, the culture medium was removed and replaced for another 48 h by serum-free DMEM for the cultures of Vero cells. The number of living cells was determined using the MTT assay at wavelength of 550 nm.