OPTIMIZATION OF BACTERIAL CELLULOSE PRODUCTION IN APPLE JUICE MEDIUM BY USING RESPONSE SURFACE METHODOLOGY (RSM)

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Thesis submitted in fulfillment of the requirements
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SUPERVISOR'S DECLARATION

I hereby declare that I have checked this thesis and in my opinion, this thesis is adequate in terms of scope and quality for the award of the degree of Bachelor of Chemical Engineering (Biotechnology)

Signature	……..……
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Position	Lecturer
Date	25 January 2012

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I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Date 25 January 2012

In The Name of Allah, Most Gracious, Most Merciful

Love special dedicate to…

Special inspiring and special encouraging of my lovely parent: Mohd Nazir Bin Sulaiman and Norihan Binti Osman;
My siblings, and also my truly best friends,
Those who has influenced my life on the right course

Thank you so much

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ABSTRACT

Acetobacter xylinum is a type of acetic acid producing bacteria that can synthesis bacterial cellulose from carbohydrates. Bacterial cellulose that produced has high purity, high water holding capacity, good mechanical strength, elasticity, high crystallinity and high porosity compare to plant cellulose. This research was using apple juice as the high potential carbon sources to replace the pure carbon sources as the substrate for the synthesis of bacterial cellulose. The objective of this study was to optimize bacterial cellulose production in apple juice medium by using Response Surface Methodology (RSM). The research will be conducted by using 5 samples with difference temperature (28ºC, 29ºC, 30ºC, 31ºC and 32ºC) , 5 sample with difference in pH (4, 5, 6 ,7 and 8) and 5 sample with different medium concentration (60 %, 70%, 80%,90% and 100%). Each sample contains 100mL of medium in 250mL conical flask and incubated in incubator for 5 days. The bacterial cellulose film produced by *Acetobacter Xylinum* was treated with 1% Natrium Hydroxide (NaOH) for 1 day and then washed with Deionized water to neutralize the bacterial cellulose. The result showed that the medium concentration, pH and temperature of cultivation were affected the production yield of bacterial cellulose. By using response surface methodology (RSM), the optimum condition for bacterial cellulose production was 95% (v/v) for medium concentration, pH 5.95 and cultivation temperature at 30.3ºC. The film then was analyzed by using Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscope (SEM). By using FTIR the hydrogen bonds (-OH) of bacterial cellulose was determined while by using SEM the interwoven strands, ribbon-like (microfibrils) of bacterial cellulose structure was observed. Thus, the optimum conditions for bacterial cellulose production can be determined by using RSM.

ABSTRAK

Acetobacter xylinum adalah sejenis asid asetik bakteria yang boleh sintesis selulosa bakteria daripada karbohidrat. Selulosa bakteria yang dihasilkan mempunyai ketulenan yang tinggi, keupayaan memegang air yang tinggi, kekuatan mekanikal yang baik, keanjalan, dan keliangan yang tinggi berbanding dengan selulosa tumbuhan. Kajian ini menggunakan jus epal sebagai sumber karbon yang tinggi yang berpotensi untuk menggantikan sumber karbon tulen sebagai substrat untuk sintesis selulosa bakteria. Objektif kajian ini adalah untuk mengoptimakan pengeluaran selulosa bakteria dalam medium jus epal dengan menggunakan Respon Kaedah Permukaan (RSM). Penyelidikan telah dijalankan dengan menggunakan 5 sampel dengan perbezaan suhu (28 º C, 29 C º, 30 º C, 31 C º dan 32 C º), 5 sampel dengan perbezaan pH (4, 5, 6, 7 dan 8) dan 5 sampel dengan kepekatan medium yang berbeza (60 %, 70%, 80%, 90% dan 100%). Setiap sampel mengandungi 100ml isipadu sampel di dalam kelalang kon 250ml dan dibiarkan di dalam inkubator selama 5 hari. Filem selulosa bakteria yang dihasilkan oleh *Acetobacter xylinum* telah dirawat dengan 1% Natrium Hidroksida (NaOH) selama 1 hari dan kemudian dibasuh dengan Deionized water (DI) untuk meneutralkan selulosa bakteria. Hasilnya menunjukkan bahawa kepekatan media, pH dan suhu fermentasi memberi impak kepada hasil pengeluaran selulosa bakteria. Dengan menggunakan kaedah respon permukaan (RSM), keadaan optima untuk pengeluaran selulosa bakteria adalah 95% (v / v) bagi kepekatan media, pH 5.95 dan pada suhu 30.3 º C. Filem ini kemudian telah dianalisa dengan menggunakan Spektroskopi Fourier Transform Infrared (FTIR) dan Pengimbasan Mikroskop Elektron (SEM). Dengan menggunakan FTIR ikatan hidrogen (-OH) selulosa bakteria telah dapat dikenalpasti manakala dengan menggunakan SEM,struktur selulosa seperti lembar terjalin, pita (microfibrils) telah berjaya diperhatikan. Oleh itu, kondusi yang optimum untuk penghasilan selulosa bakteria telah berjaya ditentukan dengan menggunakan RSM.

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LIST OF SYMBOLS

°C	Degree Celcius
cm	Centimeter
cm^{-1}	Per centimeter
g	Gram
IR	Infrared
ml	Mililiter
%	Percent

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BC	Bacterial cellulose
CCD	Central Composite Design
CSL	Corn Steep Liquor
DI	Deionizer
FTIR	Fourier Transform Infrared Spectroscopy
HS	Hestrin and Shramm
NaOH	Sodium Hydroxide
RSM	Response Surface Methodology
SEM	Scanning Electron Microscope
V/V	Volume per volume

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Bacterial cellulose is the most abundant biopolymer, that produced by some bacteria which has unique structural and mechanical properties and is highly pure as compared to plant cellulose. The molecular formula of bacterial cellulose $(C_6H_{10}O_5)_n$ is same with plant cellulose, but their physical and chemical features are different. Bacterial cellulose is extremely pure and exhibits a higher degree of polymerization and crystallinity than the fibrous polymer obtained from plant sources in which the cellulose fibrils are embedded with lignin, hemicellulose and waxy aromatic substances (Jonas and Farah, 1998).

Bacterial cellulose is synthesized by various species of bacteria belonging to the genera such as *Acetobacter, Rhizobium, Agrobacterium, Aerobacter, Achromobacter, Azotobacter, Salmonella,* and *Sarcina* (Prashant R.Chawla et al., 2008). There are many techniques for bacterial cellulose production which are stationary culture, agitated culture, cultivation in the horizontal fermenter and cultivation in the internal- loop airlift reactors. Nowadays, stationary culture has widely investigated and applied for production of some commercial cellulose product like nata de coco (Sherif M.A.S.Keshk et al., 2006). In the stationary culture condition, a thick gelatinous membrane of bacterial cellulose is accumulated on the surface of a culture medium, whereas under an agitated culture conditions cellulose can be produced in the form of fibrous suspension, irregular masses, pellets or spheres. Besides that, the cultivation medium for bacterial cellulose production mainly consists of glucose and sucrose. Common medium used for bacterial cellulose production was corn steep liquor-fructose

(CSL-Fru) and Hestrin and Shramm medium which contains mixed of chemicals and carbohydrate. These types of medium are cost effective since it used many types of chemicals in order to prepare it. Basavaraj et al. (2010) has proposed that fruits juices can play important role in commercial exploitation of bacterial cellulose by lowering the cost of medium preparation. Thus, this study was used apple juice since it has potential for enhancing the production of bacterial cellulose.

Zhiyong Yan et al. (2008) claimed that stationary culture has been widely investigated and applied for production of cellulose products such as wound care, diaphragms, foods and others. The continuous demands of plant cellulose in various uses such as paper and textile industries can lead to the depletion number of plants on earth. As the consequence, it can causes to the environmental problems such as global warming. Thus, use of bacterial cellulose can reduce the dependency on the plant cellulose.

1.2 PROBLEM STATEMENT

In previous study, most of bacterial cellulose was produced from corn steep liquor (CSL), Hestrin and Schramm (HS) medium which consisted of various types of chemicals such as glucose, yeast extract, ammonium sulphate, peptone and other additional nutrients. These types of medium are cost effective since it consists of plenty of chemicals (Takayasu Tsuchida and Fumihiro Yoshinaga, 1997). In addition, Basavaraj et al. (2010) has studied about the production of bacterial cellulose from various fruits juice which concluded that fruit juices alone as carbon source are capable to produce high yield of bacterial cellulose instead of using high cost medium. Different carbon source provide to the medium lead to different yield of bacterial cellulose production. Fructose gives the highest yield of bacterial cellulose production among of glucose, fructose, lactose and sucrose. Thus, this study was using apple juice which believed to contain high fructose. Besides that, the optimization by using Response Surface Methodology (RSM) is necessary in order to enhance the productivity of bacterial cellulose by using apple juice as medium.

1.3 OBJECTIVE

The objective of this study is to optimize the bacterial cellulose production from *Acetobacter Xylinum* by using Response Surface Methodology (RSM) and apple juice as a medium of fermentation.

1.4 SCOPE OF STUDY

i. To optimize the bacterial cellulose production by using Response Surface Methodology (RSM).
ii. To investigate the optimum pH from 4-8, temperature from 28-32°C, and medium concentration from 60%-100% (v/v) towards bacterial cellulose production.
iii. To analysis the bacterial cellulose characterization by using Fourier Transform Infrared spectroscopy (FTIR) and Scanning Electron Microscopy (SEM).

1.5 RATIONAL AND SIGNIFICANCE

This study will use apple juice that contains high fructose concentration which is suitable for bacterial cellulose production by *Acetobacter Xylinum*. Kiyoshi Aso (1951) claimed that many fruit juices were rich in carbohydrates, proteins, and trace elements thus, it can be used as a substrate for the production of bacterial cellulose. The optimization of the bacterial cellulose production is to enhance the productivity of bacterial cellulose by using cheaper carbon source (fruits juice) instead of using high cost method such as CSL-Fru, HS medium and others. Based on Yang Hu and Jeffrey M.Catchmark. (2010) research, bacterial cellulose has high purity, high degree of crystallinity, high water binding capacity and high surface area which can cause it to be use in various areas in industry including papermaking, textile, pharmaceutical, medical and others. Thus, this study is believed to optimize the bacterial cellulose production by using apple juice as the medium.

CHAPTER 2

LITERATURE REVIEW

2.1 BACTERIAL CELLULOSE

Cellulose often referred as the most abundant macromolecule on earth that produced by plant. It was a type of carbohydrate that found in plant. Apart from plants,cellulose synthesis also occurs in most groups of algae, a number of bacterial species (including the cyanobacteria), and tunicates in the animal kingdom (Inder M.Saxena et al.,2005). Cellulose consists of glucose glycosidically linked in β-1-4 conformation as shown in Figure 2.1. The repeating unit of the polymer synthesis consists of two glucose molecules bonded together. Likewise, the molecular formula of bacterial cellulose $(C_6H_{10}0_5)_n$ is the same as the plant cellulose, but their physical and chemical features are different. Bacterial cellulose is preferred over the plant cellulose as it can be obtained in higher purity and exhibits a higher degree of polymerization and crystallinity index. It also has higher tensile strength and water holding capacity than the plant cellulose (L.L.Zhou et al., 2007).

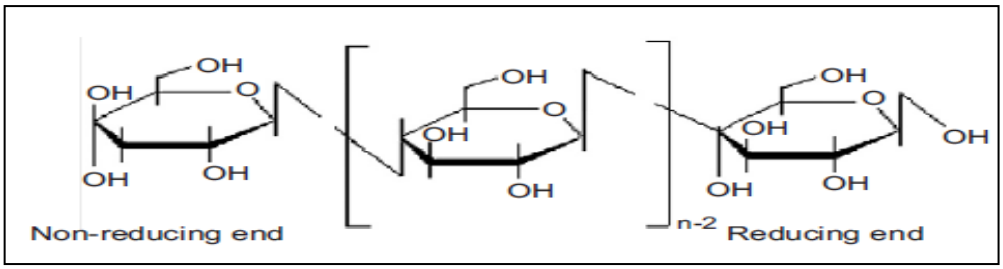


Figure 2.1. Repeating unit of cellulose

Source: R. Jonas and L.F. Farah (1998)

Bacterial cellulose or microbial cellulose exists as a basic structure known as microfibrils, which is composing of glucan chains interlocked by hydrogen bonds so that a crystalline domain is produced.

Nowadays, bacterial cellulose has been used in various areas including textile industry, paper making, food, pharmaceutical, waste treatment, broadcasting, mining and refinery (Kuan Chen Cheng et al., 2009). The study on bacterial cellulose formation by Prashant R.Chawla et al., 2008 stated that bacterial cellulose can be used in food processing as thickening and stabilizing agent. It was because of its soft texture and high fibre content. Bacterial cellulose is also been used to improve the strength properties and protects the surface of paper (Barbara Surma et al., 2008) in paper industry. Thus will help in reducing the forest depletion due to the current usage of plant derived cellulose in producing paper. Besides that,bacterial cellulose also suitable for wound healing dressing. Elvie E.Brown et al. (2007) claimed that it had been has potential to transfer of antibiotics or other medicines into the wound, while at the same time serves as an efficient physical barrier against any external infection. Apart from that, due to the unique stability, it also has been applied in the production of sound transducing membrane. The addition of bacterial cellulose will maintain the high velocity over wide frequency range and thus it becomes the best material for optimal sound transduction. However, the production of the speaker membrane by using bacterial cellulose is unsuitable to fulfill the market because of its high cost (P.R.Chawla et al., 2009).

2.1.1 Strains used for Bacterial Cellulose production

The most bacterial cellulose producers are acetic acid bacteria such as *Acetobacter Xylinum* and *Gluconacetobacter Xylinus*. Rainer Jonas and Luiz F.Farah (1997) stated that among other bacteria that can synthesis bacterial cellulose, the gram negative bacterium *Acetobacter Xylinum* is the most studied for its capacity to synthesis cellulose. *Acetobacter Xylinum* can utilize a variety of substrates for synthesizing cellulose. Sherif M.A.S Keshk (1999) reported that different substrates used can produce different yield of bacterial cellulose.

Bacterial cellulose that produced from Acetobacter xylinum is like a gel product. The product is also known as Nata that is produced by solid fermentation where it is formed and accumulated at the liquid gas interface. As the fermentation proceeds, the thickness of the gel increases, resulting in a strong fibrous structure (W.Scott Williams and Robert E.Cannon, 1989). *Acetobacter xylinum* is an extremely aerobic bacterium, thus vigorous shaking should be used to supply enough oxygen. However, due to the shear sensitive nature of the microorganisms, no cellulose product can be produced under such condition. The gel can be only obtained in static culture condition (Yoong Kook Young et al.,1997).

Acetobacter xylinum had been used as the strain to produce bacterial cellulose since years ago. It is because *Acetobacter xylinum* is a gram-negative bacterium, and it is unique in its prolific synthesis of cellulose. It produces bacterial cellulose in aerobic condition. *Acetobacter xylinum* also an acetic microbe that growth well in acidic condition of broth culture and involves in a fermentation process to convert glucose to cellulose. Gluconic, acetic or lactic acid is produced by *Acetobacter xylinum* in fermentation process and caused the pH of the medium to decrease from pH 6 to pH 4 in culture medium and at the same time the yield of cellulose decrease in fermentation (Yoong Kook Young et al., 1997). However, *Acetobacter xylinum* is still growth because it is a type of acetic microbe. In alkaline condition, *Acetobacter xylinum* will grow slowly, and bacterial cellulose yield will decrease (G.Z.Pourramezan et al., 2009). Iuliana Spiridon et al. (2010) stated that a single *Acetobacter xylinum* cell was capable of polymerizing 200 000 glucose molecules per second into β-1,4-glucan chains, which were then excreted into the surrounding medium forming ribbon, like bundles of microfibrils. The crystalline fibres produced are resembled in width and structure of average fibrils form of many plants and algae. The fibres are formed in the membrane by cellulase synthase and consequently, secreted from a row of 50 to 80 pores, like synthetic sites along the longitudinal axis of the cell (Housni Ei-said et al., 2008).

Acetobacter Xylinum has been applied as a model microorganism for basic and applied studies on cellulose. It is because of its ability to produce high levels of polymer from a wide range of carbon and nitrogen sources (Zhiyong Yan et al., 2008). It is a rod-shaped, aerobic, gram negative bacterium that produces cellulose in the form of

interwoven extracellular ribbons. This bacterium grows and produces cellulose from a wide variety of substrates. Various strains used to produce bacterial cellulose is illustrated in Table 2.1 where *Acetobacter xylinum* is the most strain that can produce cellulose using variety of substrates.

Table 2.1: Different strains used for bacterial cellulose production

Microorganism	Carbon source	Supplement	Culture time	Yield/(g/L)	Reference
A. xylinum BRC 5	glucose	ethanol, oxygen	50 h	15.30	(75)
G. hansenii PJK (KCTC 10505 BP)	glucose	oxygen	48 h	1.72	(20)
G. hansenii PJK (KCTC 10505 BP)	glucose	ethanol	72 h	2.50	(21)
Acetobacter sp. V6	glucose	ethanol	8 day	4.16	(44)
Acetobacter sp. A9	glucose	ethanol	8 day	15.20	(47)
A. xylinum BPR2001	molasses	none	72 h	7.820	(52)
A. xylinum BPR2001	fructose	agar oxygen	72 h	14.10	(64)
A. xylinum BPR2001	fructose	agar	56 h	12.00	(64)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	oxygen	52 h	10.40	(68)
Acetobacter xylinum ssp. *sucrofermentans* BPR2001	fructose	agar oxygen	44 h	8.70	(68)
Acetobacter xylinum E25	glucose	no	7 day	3.50	(78)
G. xylinus strain (K3)	mannitol	green tea	7 day	3.34	(46)
Gluconacetobacter xylinus IFO 13773	glucose	lignosulphonate	7 day	10.10	(48)
Acetobacter xylinum NUST4.1	glucose	sodium alginate	5 day	6.00	(65)
Gluconacetobacter xylinus IFO 13773	sugar cane molasses	no	7 day	5.76	(53)
Gluconacetobacter sp. RKY5	glycerol	no	144 h	5.63	(59)
Co-culture of *Gluconacetobacter* sp. st-60–12 and *Lactobacillus mali* JCM1116	sucrose	no	72 h	4.20	(60)

Source: P. R. Chawla et al (2009)

2.1.2 Cultivation Medium for *Acetobacter Xylinum*.

The fermentation medium contains carbon, nitrogen and other macro and micronutrients required for the growth of organism. *Acetobacter xylinum* can be grown in a complex medium contain glucose. A complex medium will also apply amino acids and vitamin C to enhance the cell growth and production. *Acetobacter Xylinum* needs a carbon source to growth. From the research conducted by G.Z.Pourramezan et al., (2009) it claimed that glucose and sucrose usually were used as carbon source for cellulose production besides other carbohydrates such as fructose, maltose and xylose. Jung Wook Hwang (1990) reported that using glucose as the carbon source could

decrease the production of cellulose since the pH of the medium will decrease due to the gluconic acid formation from the glucose itself. Table 2.2 tabulated various carbon sources used for cellulose production.

Table 2.2: Bacterial cellulose production from different carbon sources

Carbon source	Final pH	Yield (%)*	Consumption (%)	Cellulose Yield (%)**	Production Efficiency (%)***	Crystalinity Index (%)
Blank	6.3	22	-	-	-	-
Galactose	5.1	24				
Glucose	3.9	100	**97.0**	**8.4**	**8.7**	**88**
Fructose	5.6	95	**51.9**	**7.9**	**15.3**	**86**
Mannose	4.7	24				
Ribose	5.4	42				
Rhamnose	5.8	22				
Sorbose	5.7	23				
Xylose	4.6	38				
Lactose	6.3	22				
Trehalose	6.0	52				
Saccharose	5.9	69				
Maltose	6.1	25				
Ethanol	4.1	25				
Methanol	6.3	22				
Inositol	5.3	85	**94.7**	**7.4**	**7.8**	**75**
Glycerol	5.5	155	**45.4**	**13.0**	**28.7**	**78**

Source: Sherif M.A.S.Keshk and Kazuhiko Sameshima (2005)

Hoong Joo Son (2003) studied about bacterial cellulose production by *Acetobacter sp*.V6 in synthetic media under shaking culture condition. The synthetic media containing 1.5 percent glucose, 0.2 percent $(NH_4)_2SO_4$, 0.3 percent KH_2PO_4, 0.3 percent $Na_2HPO_4.12H_2O$, 0.08 percent $MgSO_4.7H_2O$, 0.0005 percent $FeSO_4.7H_2O$,0.0003 percent H_3BO_3, 0.00005 percent Nicotinamide, and 0.6 percent ethanol. From the research, 4.16 g/L of bacterial cellulose was produced after 8 days of cultivation at 200 rpm. This production was higher than using Hestrin and Shtamm medium.

Yasushi Sugano (2000) investigated the bacterial cellulose production by *Acetobacter xylinum* BPR 2001 in corn steep liquor fructose medium. The medium consists of 20 ml fructose, 40 g $KH_2\ PO_4$, 1 g $MgSO_4.7H_2O$,0.25g$(NH_4)_2SO_4$,3.3 g $FeSO_4.7H_2O$, 3.6 mg $CaCl_2.2H_2O$, 14.7 mg $NaMoO_4.2H_2O$, 2.42 mg $ZnSO_4.7H_2O$, 1.73

mg $MnSO_4.5H_2O$, 1.39 mg $CuSO_4.5H_2O$ and 10 ml of vitamin solution. The experiment produces 12.8 g/L of bacterial cellulose. These findings show that different culture medium will produce different bacterial cellulose yield.

2.2 APPLE JUICE

Apples are obtained from the medium sized tree belonging to the *rosaceae* family. Scientific name of apple is *Malus domestica*. Apple fruit features oval or pear shape and the outer skin has different colors depending upon the cultivar type. Internally, the juicy pulp has an off white to cream in color and has mixed of mild sweet and tart taste. The presence of fructose, glucose and sucrose in apple juice has long been established. Kiyoshi Aso and Kazuo Matsuda, (1951) reported that fructose had the largest content in apple juice depending on the maturity of the apple and days of storage. The high amount of sugar content in apple juice is suitable for some bacterium growth such as *Gluconobacter* and *Acetobacter* species. J.D MacMillan and Sheiman, (1974) claimed that apple can be extracted or pressed in order to get the juice. Figure 2.2 shows the spectrum of components in apple juice.

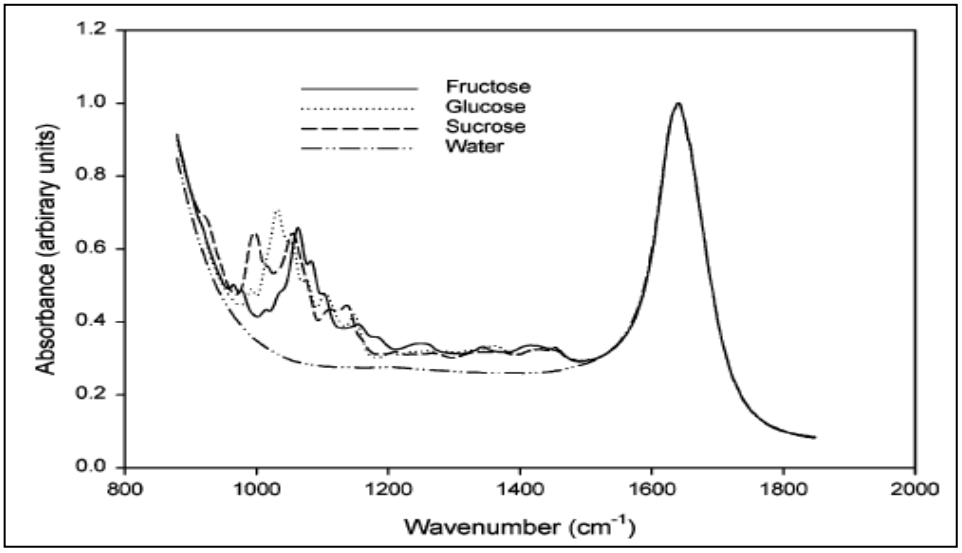


Figure 2.2: Spectra of the major components of apple juice (water, fructose, glucose and sucrose)

Source: J. F. Daniel Kelly and Gerard Downey (2005)

2.3 RESPONSE SURFACE METHODOLOGY (RSM)

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing certain process. Basically, Response Surface Methodology (RSM) is a systematic approach that can be obtained by using an inverse process of first, specifying the criteria and then computing the best design according to a formulation. RSM encompasses a point selection method to determine optimal settings of the design dimensions. It has important applications in the design, development and formulation of new products as well as in the improvement of existing product designs. RSM which includes factorial design and regression analysis can build models to evaluate the effective factors and study their interaction and select the optimum conditions in a limited number of experiments (Chauhan and Gupta, 2004). A.Jagannath et al. (2008) studied on the optimization of bacteria cellulose production by *Acetobacter xylinum* where the increase in thickness of nata-de-coco yield was after optimization. The production of bacteria cellulose from coconut water as a substrate increased by pH 4.0 with 10% sucrose and 0.5% ammonium sulphate concentration by using of Response Surface Methodology (RSM).

Optimization of bacteria cellulose production in a batch reactor by using *Acetobacter xynilum* was conducted by (Milda E. Embuscado et al., 2009). The effect of several factors such as fructose and sucrose concentrations, pH and temperatures of incubation were evaluated and all four factors affected cellulose yield significantly. Four-factor central composite design was used in Response Surface Methodology (RSM) to determine the relationship of four factors (fructose and sucrose concentrations, pH and temperature of incubation) to the response, cellulose yield (in g of crude cellulose/L of medium). The optimum fermentation conditions were obtained throughout the study. It indicates that the four parameters fructose concentration,glucose concentration,pH and temperature of incubation had their relation in order to obtain the optimum value of each single parameter. Upon verification, the predicted cellulose yield (13.24 g/l) was found to be very close to the average experimental yield (12.67 g/l), indicating that the mathematical model obtained was an adequate predictor of cellulose yield.

2.4 FOURIER TRANSFORM INFRARED (FTIR)

Movasaghi et al. (2008) claims that Fourier Transform Infrared (FTIR) is the analysis technique that provides information about the chemical bonding or structure of materials, whether organic or inorganic. This technique offers a non destructive alternative to chemical measurement technique for qualitative characterization. FTIR consists of four arms. The first arm contains a source of infrared light, the second arm contains a stationary mirror, the third arm contains a moving mirror, and the fourth arm is open. The beam splitter at the intersection of the four arms is design to transmit half of the radiation that impinges upon it, and reflects half of it. As a result, the light transmitted by the beam splitter strikes the fixed mirror, and the light transmitted reflects by beam splitter strike the moving mirror. Then, the two light beams recombine at the beam splitter, and leave the interferometer to interact with sample and strike the detector (Smith et al.,1996). The Fourier Transform Infrared analyzes the cellulose based on the chemical bonding that present in the cellulose. The whole and expanded FTIR spectra revealing the characteristics absorption band of bacteria cellulose. The characteristics bands that appeared are list in Table 2.3.

Table 2.3: Characteristics bands of cellulose bonds

Chemical bonding	BC peak(cm)	References
Carbonyl group(C=O)	1650 cm-1	Iuliana et al., 1989
C-O-H	672@711 cm-1	L.L.Zhou et al., 2007
C-H Bonding	i)1430-1290 cm-1 ii)2942 cm-1	i) Housni et al., 2008 ii) Saharman et al, 2005
C-0 strecthing at C3	1060 cm-1	L.L.Zhou, et al., 2007
C-O stretching at C6	1030 cm-1	Elvie E.Brown et al., 2007
C-C stretching at C6	1030 cm-1	L.L.Zhou, et al., 2007
C-O-C stretching at b-glycosidic linkage	i)1160@900 cm-1 ii) 1160 cm-1	i)L.L.Zhou, et al., 2007 ii)Muenduen, et al., 2008

Source: Saharman Gea et al. (2011)

Referring to Table 2.3, the change or rearrangement of the cellulose structure cause the absorbance peak of the wave number is either decreased or shifted to higher or lower value. Sun et al., 2008 stated that when an enzyme, diluted acid, and sodium hydroxide were treated into the cellulose, some of the chemical bond on the surface of cellulose will be broken down in the reaction and the hidden internal chemical bond will be exposed. For example, the stretching absorbency increased when the effect of acid was first appeared on the surface and amourphous zone, thus the hydrogen bonds broken and more bond type's C-OH, C-O-C, and C-C were exposed. Some of the absorbance peak will change either decrease or shifted to a greater or lower wave number when the cellulose structure is changing.

2.5 SCANNING ELECTRON MICROSCOPE (SEM)

The Scanning Electron Microscopy is an instrument that reveals the sample's information such as chemical composition, crystalline structure and crystalline orientation. Weihua Tang et al. (2009) reported that they used scanning electron microscope (SEM) to observe the structure of bacterial cellulose. It showed a smooth surface with no visible pores at magnification 10000X as illustrated in Figure 2.3. Bacterial cellulose has a three-dimensional network which retained a lot of water and transportation of nutrients. Besides that, Zhiyong Yan (2008) was also used scanning electron microscope to observe the bacterial cellulose microfibrils from different types of cultivation. The agitated bacterial cellulose structure was bands twist and curl apparently while in static bacterial cellulose it microfibrils were straighter.

Muenduen Phisalapong and Nirun Jatupaiboon (2008) studied on a surface morphological of bacterial cellulose with and without addition of chitosan by using scanning electron microscope (SEM). By adding chitosan, the bacterial cellulose structure become denser compared to the bacterial cellulose without chitosan addition.

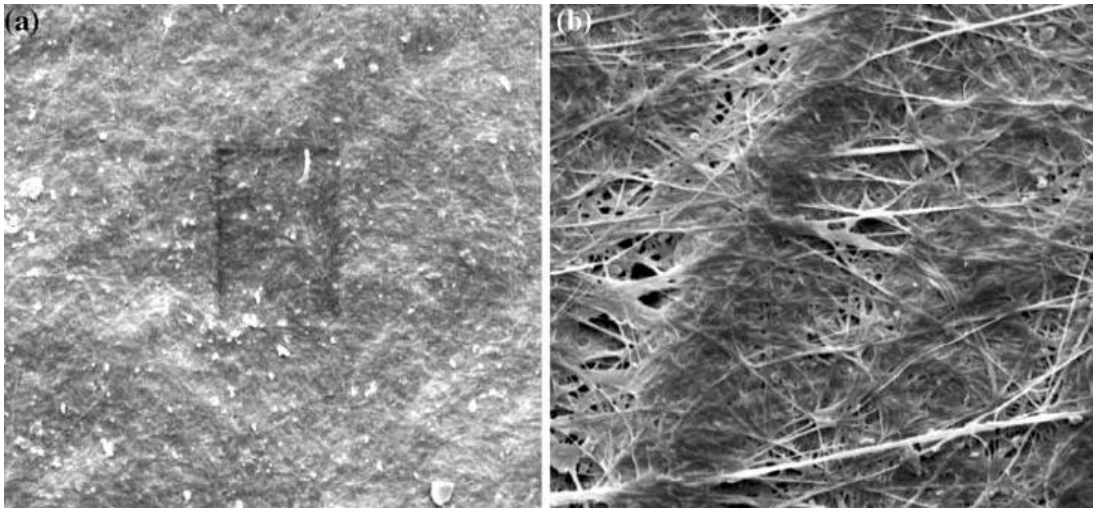


Figure 2.3: Microfibrial structure under Scanning Electron Microscope

Source: Weihua Tang et al. (2006)

Besides that, a study about treated and untreated surface of bacterial cellulose has been studied by Saharman et al. (2011). In this report, the researchers used scanning electron microscopic at 5000X magnification in order to observe the bacterial cellulose structure. The surface of untreated bacterial cellulose was opaque meaning that it is impossible to study the internal structure of the untreated BC network directly.

CHAPTER 3

METHODOLOGY

3.1 INTRODUCTION

This chapter presents the methodology used to produce bacterial cellulose by *Acetobacter Xylinum* using apple juice as the medium. First the bacterial cellulose will be obtained by fermentation using bacteria *Acetobacter xylinum* as the strain and apple juice as the culture broth. Then, the optimum variables such as temperature, pH, and medium concentration will be obtained by using Response Surface Methodology (RSM).

3.2 MATERIALS AND APPARATUS

The stock culture of *Acetobacter xylinum* was supplied by Malaysian Agricultural Research and Development Institute (MARDI), Serdang Selangor. Apple fruits were purchased from a market nearby university. Other chemicals such as Ammonium Sulphate ($NHSO_4$), Sodium Hydroxide (NaOH), Acetic Acid, distilled water, yeast extracts, bectopeptone, Na_2HPO_4, citric acid, Magnesium Sulphate,$MgSO_4.7H_2O$, Sucrose and Glucose were be purchased from Merck company.

3.2.1 Preparation of Bacterial Cellulose

The mother culture medium for the inoculum that was used throughout this study was coconut water that consisted of 5.0% glucose, 0.5% ammonium sulfate $(NH_4)_2SO_4$ and 1.0% acetic acid. The medium was sterilized in an autoclave at 121°C for 30 minutes. 100 mL of a stock culture was inoculated into 1000 mL of medium and incubated at 30°C for 1 week in static culture. After 1 week a white membrane like gel formed at the medium interface. The membrane was purified by washing with deionized (DI) water and then treated with 1% (w/v) of sodium hydroxide (NaOH) solution at room temperature for 1 day to remove bacterial cells followed by a rinse with DI water until the pH become neutral.

3.3 METHODS

At first inoculum was prepared in the 500ml beaker. For the medium of cultivation, apple fruits were blended by using blender. 2.5 g of ammonium sulphate, NH_3SO_4, 2 g of glucose were inserted into the apple juice as the fermentation medium. The medium then was autoclaved at 121ºC for 30 minutes. Then, for the cultivation, 10 mL of inoculum was mixed with 100 mL of fermentation medium and incubated at different values of variables for 5 days. The bacterial cellulose pellicle formed on the surface of the cultivation medium was treated with 1% (w/v) natrium hydroxide solution and washed with deionized (DI) water. The wet bacterial cellulose was dried in the oven at 60 ºC to 80 ºC for 30 minutes and analyzed by using Fourier Transform Infrared (FTIR) and Scanning Electron Microscopy (SEM).

3.3.1 Fermentation Process

The static fermentation of bacterial cellulose was carried out inside a 250 ml beaker, which contained 100 ml of medium and 10 ml of stock culture. The medium used in this study was apple juice with additional of some chemicals. To complete the process, the fermentation medium was incubated at various temperatures, pH and concentration of a medium. A further explanation will be provided in next subtopic. The fermentation's process was conducted for 5 days inside the incubator, for the required temperature for each set.

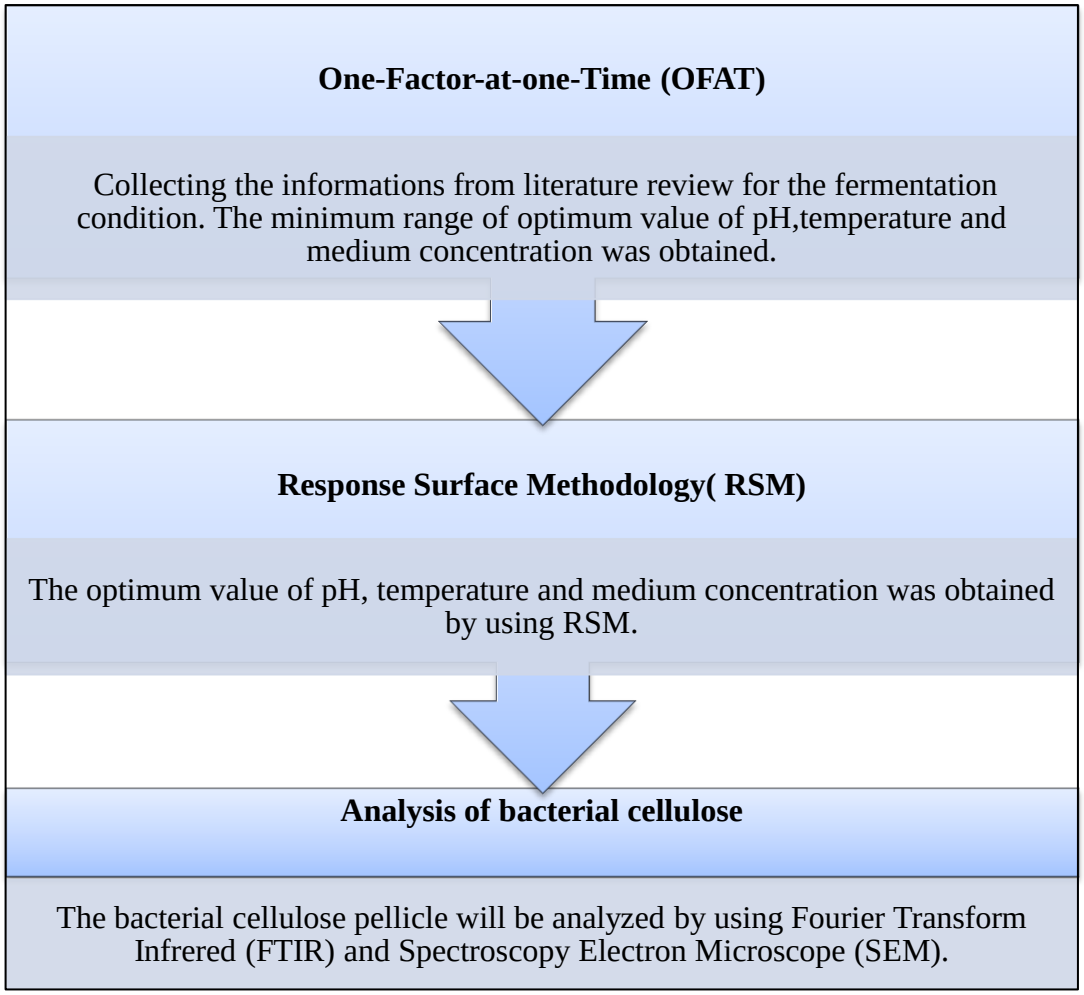


Figure 3.1: Flow process of experimental procedures

3.4 ONE-FACTOR-AT-ONE-TIME (OFAT)

One factor at one time is a process where the researchers run the experiments in order to get the minimum range of every single variable that needed to be determined. The variables involved in this study were pH, temperature and medium concentration.

3.4.1 Temperature

Most of the previous research conducted used to cultivate the bacterial cellulose at 30ºC. According to Kuan Chen Cheng et al. (2009) the fermentation was run at 30ºC and 7.05g/L of bacterial cellulose was produced. Other than that, Barbara Surma-Slusarska (2008) reported that the high yield of bacterial cellulose at 30ºC followed by 25ºC and low yield at 35ºC. Based on Basavaraj S.Hungund and S.G.Gupta,the maximum production of bacterial cellulose was at 28ºC. From the above observations it can be conclude that the optimum condition for bacterial cellulose fermentation will be at temperature 30ºC. Thus, the range of fermentation temperature was done from 25ºC to 35ºC.

3.4.2 pH

During the fermentation, the pH of the medium might be decrease due to the conversion of glucose to gluconic acid**.** The pH needed to be adjusted to the optimum pH by using acetic acid, CH_3COOH. Takaaki Naritomi et al. (1997) reported for the fermentation condition was at pH 5. Basavaraj S.Hungund and S.G.Gupta (2002) used pH range from 4 to 7 and managed to achieve maximum bacterial cellulose production at pH 6.5. Kewei Zuo et al. (2006) were able to produce maximum bacterial cellulose at pH 5.5 in an airlift reactor. Thus, from these findings, it can be concluded that the pH range of this research was in range of 4 to 7.

3.4.3 Medium concentration

This research will use apple juice as the medium that contains 5.0% sucrose, 0.5% ammonium sulphate and 1.0% acetic acid. Based on Ong-ard Saibuatong and Muenduen Phisalaphong (2003), coconut water was used for the fermentation medium which supplemented with 5.0% sucrose, 0.5% ammonium sulfate and 1.0% acetic acid. From these findings, the medium concentration was studied at range of 60, 70, 80, 90 and 100% (v/v) respectively (J.F.Daniel Kelly and Gerard Downey, 2005).

3.5 RESPONSE SURFACE METHODOLOGY (RSM)

The optimum value of each parameter (pH, temperature, medium concentration) can be obtained by using Response Surface Methodology software. The range value from OFAT of each parameter was used in RSM software. This research contains three main parameters that need to be determined by using RSM. The most suitable mode to be used in RSM was Central Composite Design (CCD). The ranges of optimum value of parameters were inserted in Central Composite Design and a table consists of several runs of the fermentation process was provided. Table 3.1 shows the response column of CCD. A 2^3 full factorial central composite design and RSM were applied to determine the optimum value of each significant variable. By following each bacterial cellulose fermentation condition provided for each trial, the results were obtained. Finally the system gave the equation that represented the optimal bacterial cellulose production (Zhang Jian and Gao Nian-fa, 2006).

Table 3.1: Response column of CCD

	Std	Run	Block	Factor 1 A:pH	Factor 2 B:Medium Con %	Factor 3 C:Temperature C	Response 1 BC dry weight g
	8	1	Block 1	6.50	95.00	31.00	1.0214
	16	2	Block 1	6.00	90.00	30.00	1.0342
	5	3	Block 1	5.50	85.00	31.00	0.6765
	3	4	Block 1	5.50	95.00	29.00	0.8113
	13	5	Block 1	6.00	90.00	28.00	0.4122
	6	6	Block 1	6.50	85.00	31.00	0.3905
	1	7	Block 1	5.50	85.00	29.00	0.3102
	10	8	Block 1	7.00	90.00	30.00	0.254
	15	9	Block 1	6.00	90.00	30.00	0.7861
	11	10	Block 1	6.00	80.00	30.00	0.9182
	18	11	Block 1	6.00	90.00	30.00	1.2013
	20	12	Block 1	6.00	90.00	30.00	1.0994
	14	13	Block 1	6.00	90.00	32.00	0.4977
	2	14	Block 1	6.50	85.00	29.00	0.3978
	12	15	Block 1	6.00	100.00	30.00	1.4798
	17	16	Block 1	6.00	90.00	30.00	1.0562
	7	17	Block 1	5.50	95.00	31.00	0.7769
	19	18	Block 1	6.00	90.00	30.00	1.0939
	9	19	Block 1	5.00	90.00	30.00	0.6614
	4	20	Block 1	6.50	95.00	29.00	0.5596

3.6 ANALYSIS OF BACTERIAL CELLULOSE

3.6.1 Fourier Transform Infrared (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was performed by using FTIR Nicolet Avatar 370 DTGS in Universiti Malaysia Pahang laboratory. FTIR spectra will be recorded between 1100 and 4000 cm^{-1} with a piece of film dimension of 2 cm x 2cm in size. Spectral output was recorded in absorbance as a function of a wave number.

FTIR spectral pattern was analyzed and matched with known signatures of identified materials in the FTIR library.

3.6.2 Scanning Electron Microscope (SEM)

The characterization on microstructure of samples was performed by using Scanning Electron Microscope (SEM) EDX Spectrometer EVO 50 that operated at an acceleration voltage of 15kV. The surface and cross section of Scanning Electron Microscope was observed between 100x and 1000x resolution. The bacterial cellulose sample was frozen in liquid nitrogen. Then the sample was snapped vacuum dried and then sputter with gold (Saharman Gea et al. 2011).

CHAPTER 4

RESULT AND DISCUSSION

4.1 INTRODUCTION

This chapter is discussing about the result from the study. The range of optimum value for each parameter such as fermentation temperature, pH and concentration of the cultivation medium were studied by using One-Factor-At-One-Time (OFAT). Later, the studies are continued by using Response Surface Methodology (RSM) in order to obtain maximum production of bacterial cellulose from the fermentation process. The chemical and physical properties of bacterial cellulose produced from the fermentation process were analyzed by using Fourier Transform Infrared (FTIR) and Scanning Electron Microscope (SEM).

4.2 ONE FACTOR AT ONE TIME (OFAT)

4.2.1 Temperature

Cultivation temperature plays an important role for production of bacterial cellulose from *Acetobacter Xylinum*. This microbe needs energy to convert glucose molecule into cellulose, so if the temperature increase, the production will increase concurrently at temperature of 30°C (Pourramezan et al.,2009). In this study, the cultivation temperature was in range from 28°C to 32°C. Table 4.1 below showed the result from the experiment that has been run while Figure 4.1 showed the graph of dry weight BC versus cultivation temperature.

Table 4.1: Experiment result

Temperature(°C)	BC wet weight(g)	BC dry weight(g)
28	37.5341	1.0552
29	39.4436	1.1145
30	46.7330	1.8446
31	35.2631	1.0331
32	28.7342	0.9114

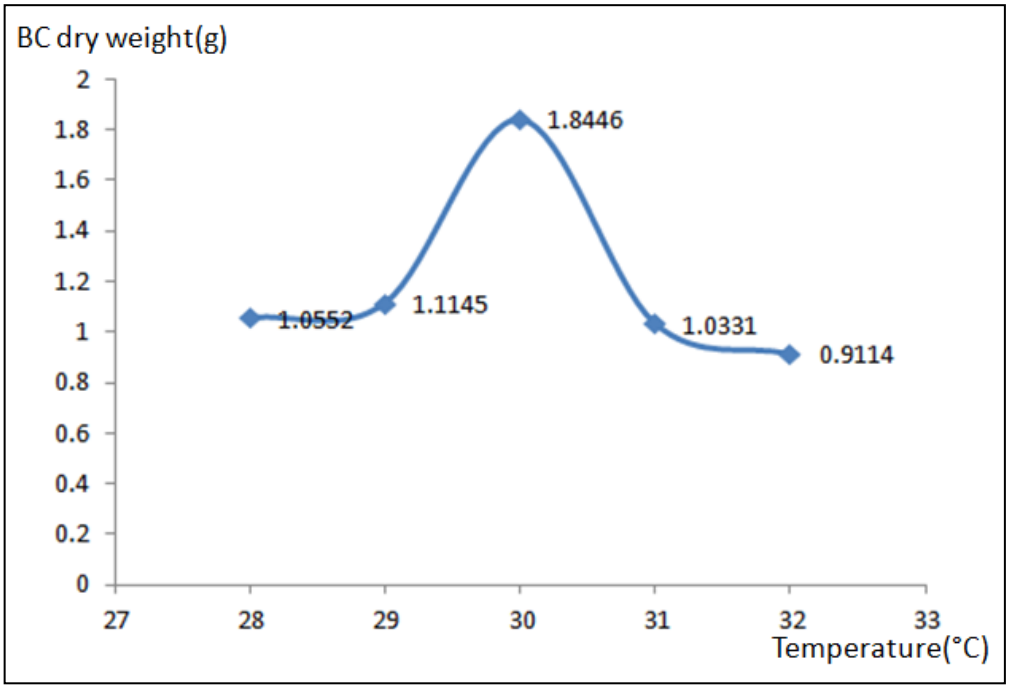


Figure 4.1: Graph BC dry weight versus temperature

From Table 4.1 and Figure 4.1 it was showed that the weight of bacterial cellulose was increased due to the increased of temperature until 30°C and then decreased at temperature above 30°C. However after 30°C, the BC weight decreased because too high temperature might cause the bacteria to die. Most of the bacteria was not resistance to heat and will die at high temperature. The weight of bacterial cellulose increases until 30°C because more energy was supplied to the bacteria to convert the glucose and fructose molecule into cellulose (A.Retgi et al.,2010).

4.2.2 pH Medium

Acetobacter Xylinum is a type of microbe that shown resistance to acidic condition. In this study, the experiment was run at pH range from 4 to 8 (Yang Hu and Jeffrey M. Catchmark. 2010). From this pH range, several experiments were done at fixed temperature and medium concentration. Thus, the results from this experiment were obtained as illustrated in Table 4.2 below.

Table 4.2: Experiment Result on pH

pH	BC wet weight(g)	BC dry weight(g)
4	0.948	0.060
5	21.545	0.487
6	29.730	0.770
7	28.389	0.745
8	25.890	0.593

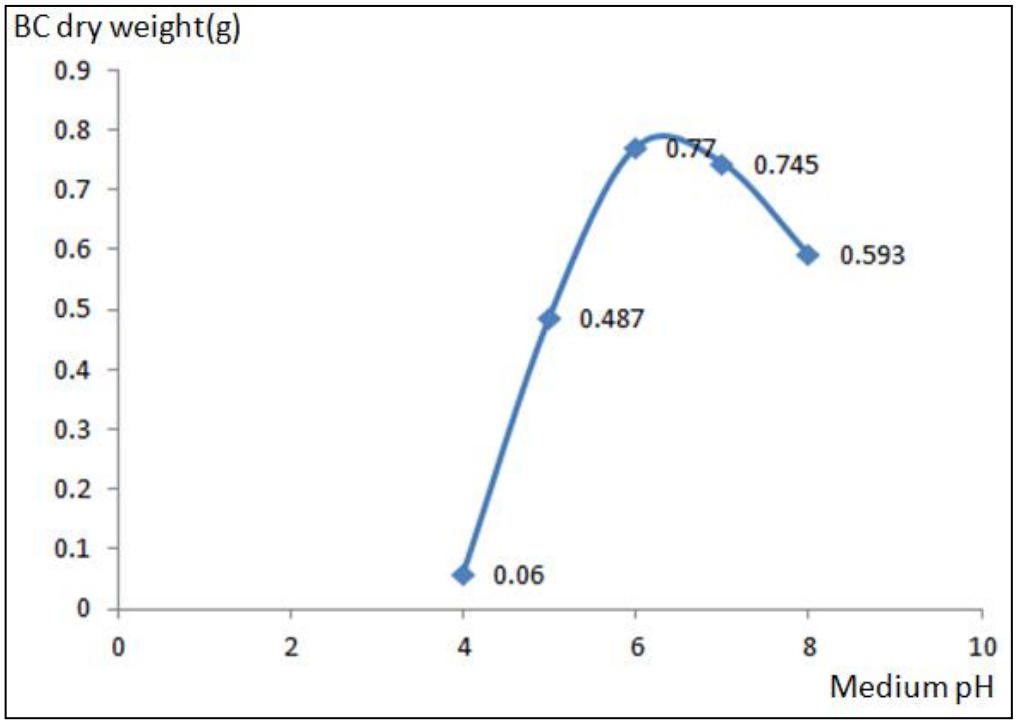


Figure 4.2: Graph BC dry weight against pH

According to Figure 4.2 above, it shows that the weight of dry BC was increased due to increased of medium pH. The optimum of BC weight was at pH 6 with 0.770g while at pH 4 the BC weight was the lowest that was 0.06g. BC dry weight increased from pH 4 to pH 6 then decreased when above pH 6. This shown that the activity of *Acetobacter Xylinum* was optimum at pH 6. At pH 7 and 8, the activity of *Acetobacter Xylinum* was slow because of the formation of gluconic acid during the cultivation time that leads to the decreased of bacterial cellulose formation (Prashant R.Chawla et al.,2008).

4.2.3 Medium Concentration

This study was using apple juice as the fermentation medium. Based on the OFAT result from the effect of incubation temperature and pH medium were used to study the effect of apple juice concentration which was the medium for the fermentation. The apple juice was added with ammonium sulphate as the nitrogen source for the bacteria growth and the apple juice concentration was measured by percentage volume per volume (v/v) of apple and distilled water. The experiment was run according to this set of medium concentration (60%, 70%, 80%, 90% and 100%) at constant pH and temperature (Young Kook Yang et al.,1999). Table 4.3 below showed the result from the experiment.

Table 4.3: Experiment result

Concentration (%)	Wet weight(g)	Dry weight(g)
60	32.407	1.358
70	36.395	1.844
80	36.102	1.759
90	48.654	2.498
100	61.138	3.630

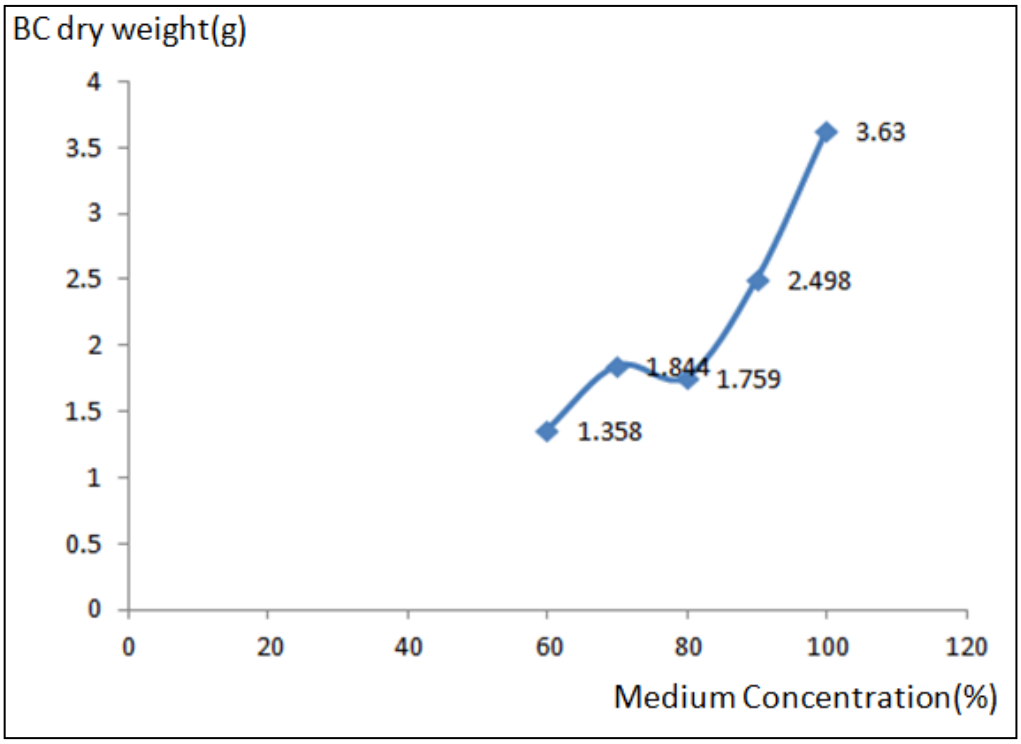


Figure 4.3: Graph BC dry weight against Medium concentration

From Figure 4.3 above it can be seen that weight of bacterial cellulose was increased from 60% to 70% and was decreased at 80% concentration. After 80% concentration, the weight of BC was increased again until 100% concentration and at this point the medium became concentrated. The increased of BC formation was due to the presence of carbon sources in the medium that caused the bacteria to convert glucose to bacterial cellulose rapidly. During the fermentation,the *Acetobacter Xylinum* converted glucose to gluconic acid. The accumulation and consumption of gluconic acid in the fermentation medium contribute to the changes of the pH medium, therefore the weight of bacterial cellulose decrease because more gluconic acid into the medium fermentation (Hwang et al.,1999).

This was proven by S.Masaoka et al. (1993) which claimed that the formation of gluconic acid as by-product in the medium decreased the pH of the medium and ultimately decreased the production of bacterial cellulose. In this report,the medium used apple juice which content high in fructose compared to glucose. In Figure 4.3,the BC production decreased from 70 to 80% concentration. At that point,the bacteria

converted all the glucose in the medium to gluconic acid. The formation of this gluconic acid caused the pH of the medium to decrease. The decreased of the pH caused to the decreased of BC production. After 80% concentration,there was no more glucose in the medium because it was fully utilized by the bacteria but only fructose remained in the medium as a carbon source. Due to this situation,the BC formation increased again until 100% concentration.

4.3 RESPONSE SURFACE METHODOLOGY (RSM)

The optimum range for each parameter had been determined from the OFAT experiment. These optimum ranges of variables were used in RSM in order to determine the optimum condition for the fermentation process. The optimum ranges for each variable were listed in Table 4.4 below.

Table 4.4: Variables range

Parameter	**Lower limit**	**Upper limit**
pH	5.5	6.5
Temperature(°C)	29	31
Medium Concentration (%)	85	95

This range was inserted into Central Composite Design (CCD) in RSM. From CCD, there were 20 experiments needed to be run in order to find the optimum point for each variable as tabulated in Table 4.5.

Table 4.5: Experiment result

	Std	Run	Block	Factor 1 A:pH	Factor 2 B:Medium Con %	Factor 3 C:Temperature C	Response 1 BC dry weight g
	8	1	Block 1	6.50	95.00	31.00	1.0214
	16	2	Block 1	6.00	90.00	30.00	1.0342
	5	3	Block 1	5.50	85.00	31.00	0.6765
	3	4	Block 1	5.50	95.00	29.00	0.8113
	13	5	Block 1	6.00	90.00	28.00	0.4122
	6	6	Block 1	6.50	85.00	31.00	0.3905
	1	7	Block 1	5.50	85.00	29.00	0.3102
	10	8	Block 1	7.00	90.00	30.00	0.254
	15	9	Block 1	6.00	90.00	30.00	0.7861
	11	10	Block 1	6.00	80.00	30.00	0.9182
	18	11	Block 1	6.00	90.00	30.00	1.2013
	20	12	Block 1	6.00	90.00	30.00	1.0994
	14	13	Block 1	6.00	90.00	32.00	0.4977
	2	14	Block 1	6.50	85.00	29.00	0.3978
	12	15	Block 1	6.00	100.00	30.00	1.4798
	17	16	Block 1	6.00	90.00	30.00	1.0562
	7	17	Block 1	5.50	95.00	31.00	0.7769
	19	18	Block 1	6.00	90.00	30.00	1.0939
	9	19	Block 1	5.00	90.00	30.00	0.6614
	4	20	Block 1	6.50	95.00	29.00	0.5596

From Table 4.5, there were 20 sets of experiment that had been generated by the Central Composite Design (CCD). Based on Table 4.5 above, the highest bacterial cellulose weight was at run 15, which produced 1.4798g of dry weight bacterial cellulose while the lowest production of bacterial cellulose was at run 8, which produced 0.254g dry weight bacterial cellulose. This was because a different set of conditions leaded to different production of bacterial cellulose. Moreover, at run 8 and run 11 the pH was set 7 and 6 respectively. The different in pH setting caused to different production of bacterial cellulose. Rainer Jonas and Luiz Farah (1998) stated that the suitable pH for the production of bacterial cellulose was below 7. Thus, it can

be concluded that high pH was not suitable for the bacterial cellulose production from *Acetobacter Xylinum.*

Besides that, the different setting for run 11 and run 13 was only for temperature of the cultivation. Run 11 was set at 30°C while run 13 was at 32°C. The different set of temperature produced different weight of bacterial cellulose. Thus, it can be concluded that high temperature was unsuitable for bacterial cellulose production (Housni El-Said et al.,2008).

Response: BC dry weight

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares]

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	1.80	9	0.20	5.39	0.0073	significant
A	*0.065*	*1*	*0.065*	*1.75*	*0.2148*	
B	*0.40*	*1*	*0.40*	*10.68*	*0.0085*	
C	*0.057*	*1*	*0.057*	*1.54*	*0.2423*	
A^2	*0.66*	*1*	*0.66*	*17.88*	*0.0017*	
B^2	*0.013*	*1*	*0.013*	*0.36*	*0.5644*	
C^2	*0.67*	*1*	*0.67*	*18.04*	*0.0017*	
AB	*4.570E-003*	*1*	*4.570E-003*	*0.12*	*0.7329*	
AC	*1.879E-003*	*1*	*1.879E-003*	*0.051*	*0.8265*	
BC	*5.848E-004*	*1*	*5.848E-004*	*0.016*	*0.9026*	
Residual	0.37	10	0.037			
Lack of Fit	*0.27*	*5*	*0.055*	*2.82*	*0.1397*	*not significant*
Pure Error	*0.097*	*5*	*0.019*			
Cor Total	2.17	19				

Figure 4.4: ANOVA for response surface quadratic model

In Figure 4.4 above showed the lack of fit was not significant. This signified that the regression model was precise in predicting the pattern of significance to the production of bacterial cellulose. Besides that, the determination coefficient (R^2) was 0.8290 as shown in Figure 4.5 which ensures an adequate credibility of the regression model. This R^2 indicated that only 17.1% of the total variations were not explained by the model. Other factors such as incubation time, type of strain used might affect the BC

production. Thus, the regression model represented the model response. This quadratic regression model was used to find the optimum condition by substituting the variables value (Zhang Jian and Gao Nian-fa,2007). To investigate the effects of the three variables on the dry weight of bacterial cellulose, the response surface methodology was used, and the tree-dimensional (3D) plot was drawn. Figure 4.6(a-c) showed the response surface plot for the variables (pH, temperature and medium concentration) interaction with the bacterial cellulose dry weight. The quadratic regression model was shown by equation (1) below.

$$\text{BC dry weight} = 1.01 - 0.064A + 0.16B + 0.06C - 0.16A^2 + 0.023B^2 - 0.16C^2 + 0.024AB + 0.015AC + 8.55\times10^{-3}BC \quad (1)$$

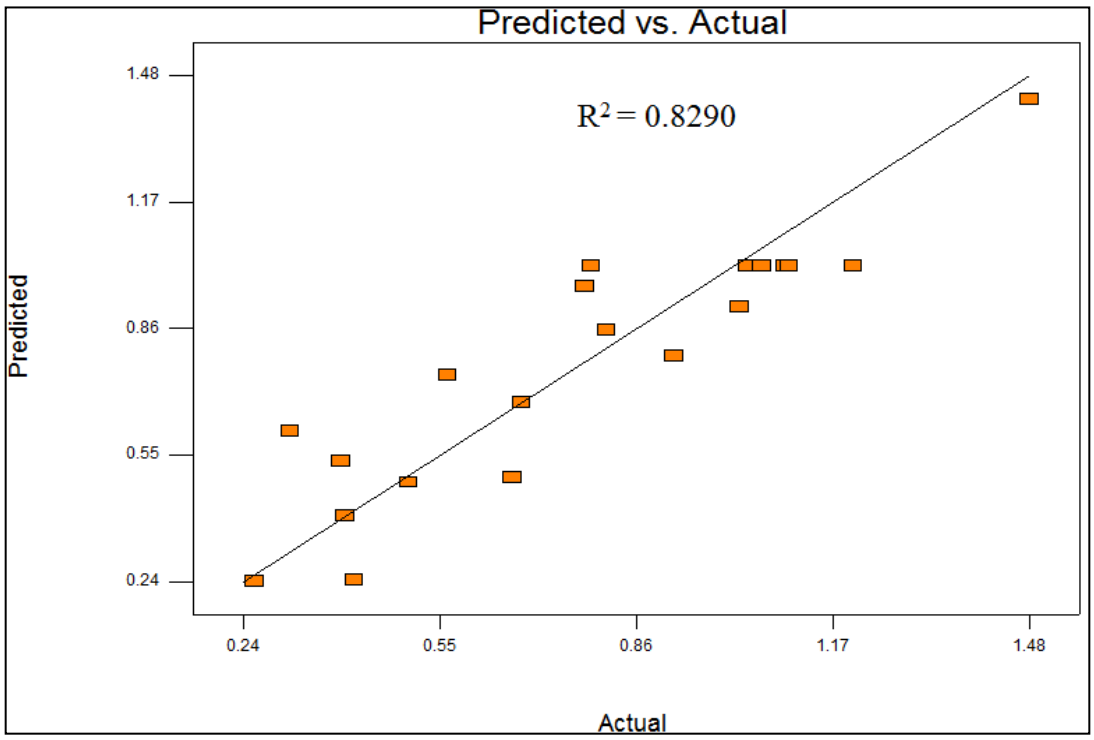


Figure 4.5: Predicted versus actual value graph

Figure 4.5 above showed the graph of predicted versus actual value for BC dry weight. It tabulated the points of each of the 20 experiment that had been run. The predicted versus actual graph represented the behavior of the bacterial cellulose

production which showed that less error with the actual value that represented with the linear plot. Based on the plot it can be concluded that the actual value was proportional with the predicted value since the error was less. Thus, this design was able to optimize the bacterial cellulose production.

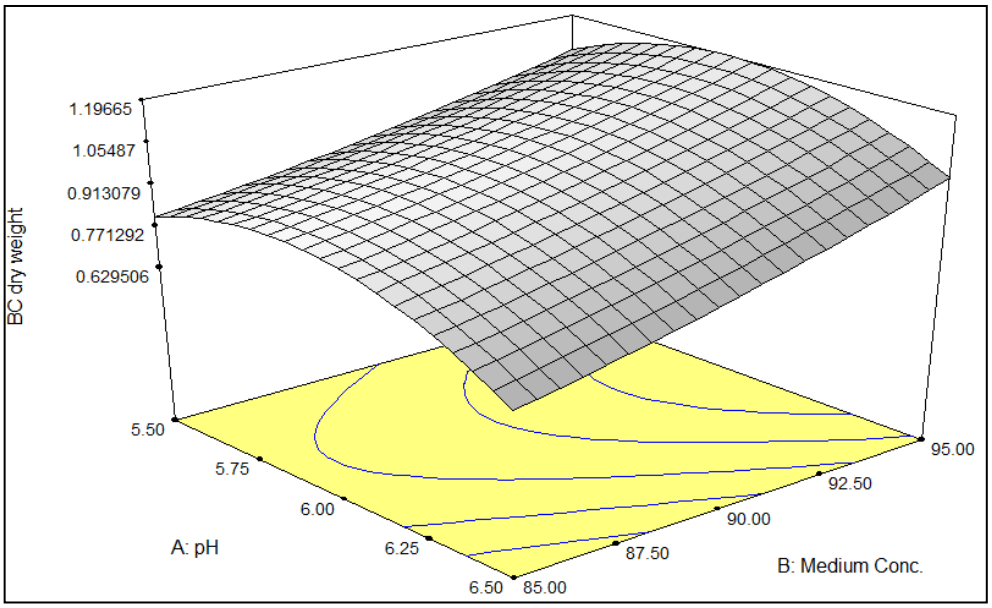


Figure 4.6(a): pH versus medium concentration

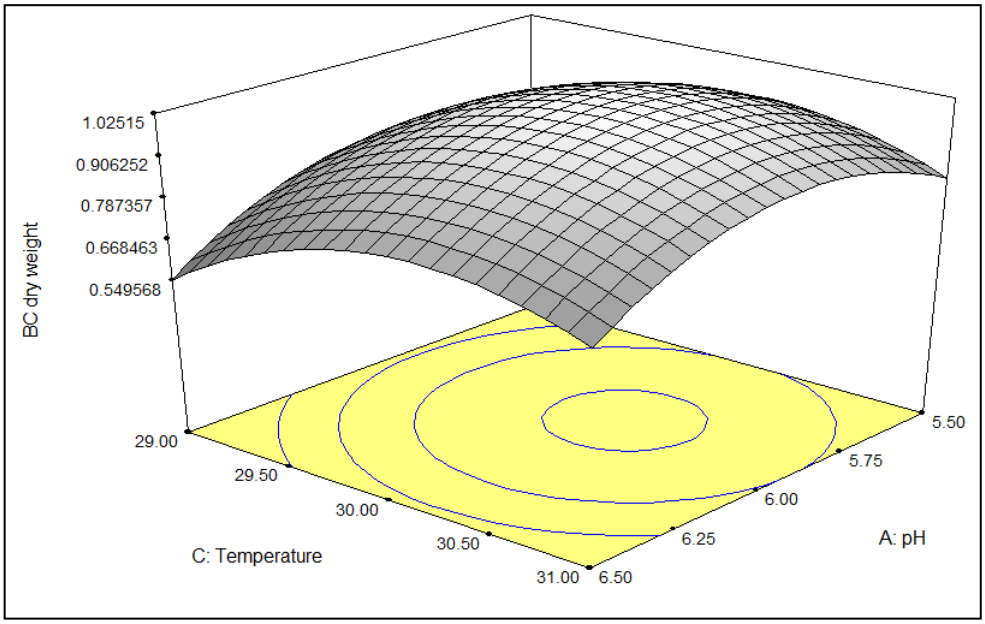


Figure 4.6(b): Temperature versus pH

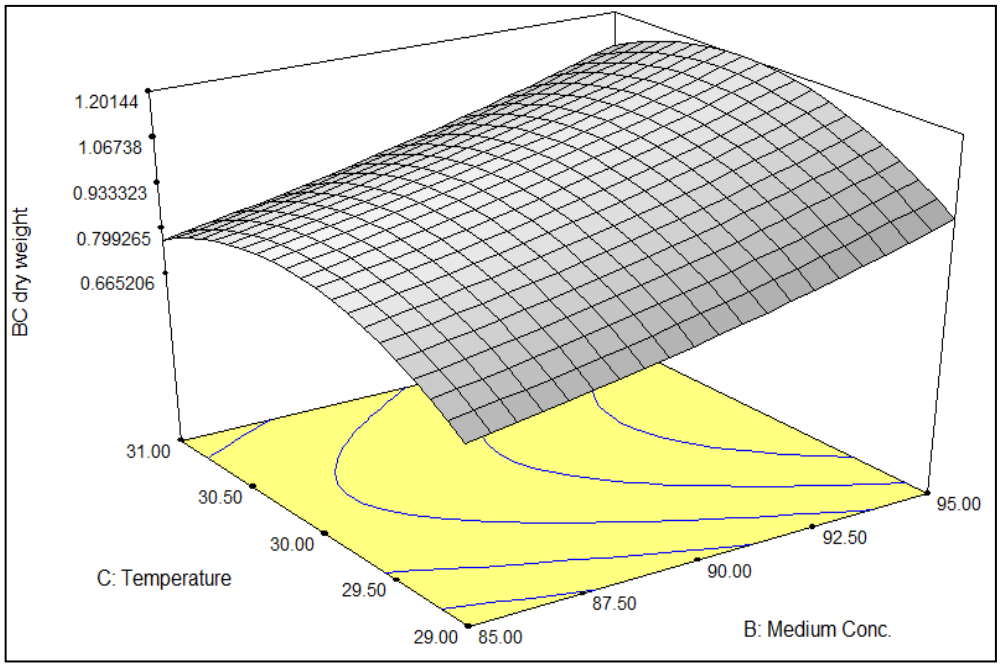


Figure 4.6(c): Temperature versus medium concentration

Figure 4.6(a-c) showed the interaction between the variables (pH, temperature and medium concentration) to the BC dry weight. The curvature shape for each graph was reflected by the significant effect of the variables (AB, AC and BC) towards the bacterial cellulose production. This different type of plot was due to the different in the values substituted into the quadratic regression model (Jun Wang et al., 2009).

Figure 4.6(b) showed the interaction between pH and temperature of cultivation. From the figure, it showed that the maximum response was at pH 6 and temperature 30°C. From this it can be concluded that the coefficients of the regression equation determined from the experiments data was resulted with maximum response surface which indicated that the interaction of AB were believed to enhance the optimization of the bacterial cellulose production significantly.

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
pH	is in range	5.5	6.5	1	1	3
Medium Conc.	is in range	85	95	1	1	3
Temperature	is in range	29	31	1	1	3
BC dry weight	maximize	0.254	1.4798	1	1	3

Solutions

Number	pH	Medium Conc.	Temperature	BC dry weight	Desirability	
1	5.94	95.00	30.20	1.20353	0.775	Selected
2	5.95	95.00	30.24	1.20332	0.774	
3	5.95	95.00	30.29	1.20237	0.774	

Figure 4.7: Optimized condition for bacterial cellulose production

From Figure 4.7, there were 3 sets of experiment that have been generated by RSM. The results from the experiments were shown in Table 4.6 below.

Table 4.6: Optimized Solution Result

Experiment	pH	Temperature (°C)	Medium Concentration (%)	BC dry weight (g)	Percentage Of Error (%)
1	5.94	30.2	95	1.2173	1.14
2	5.95	30.2	95	1.3065	8.57
3	5.95	30.3	95	1.1986	0.31

From Table 4.6 above, the percentage of error between RSM value and experimental value was below 10%. This was an acceptable result since the percentage of error should be less than 10%. Thus, these conditions were accepted as the optimum condition for bacterial cellulose production. The percentage of error was calculated by the following equation (2).

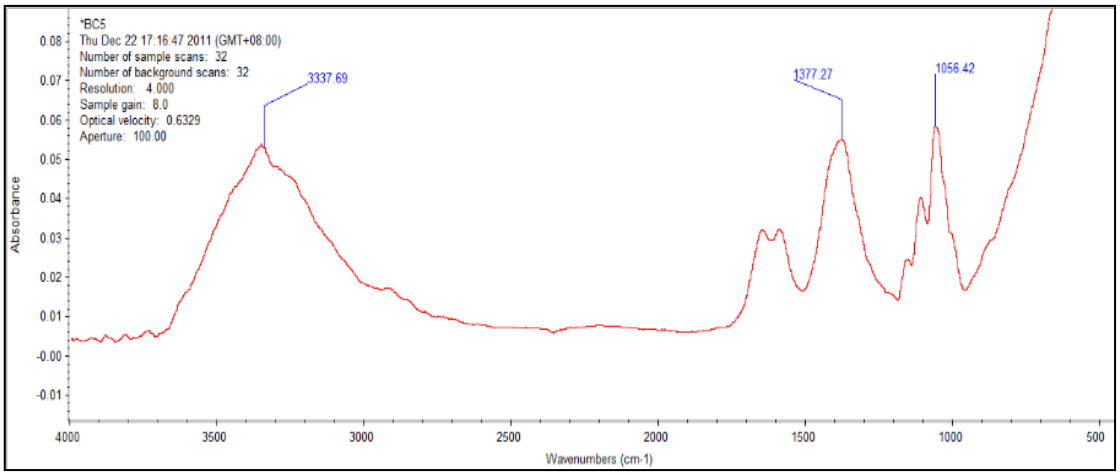
$$\text{Percentage of error}(\%) = \frac{\text{Actual yield} - \text{Predicted Yield}}{\text{Predicted Yield}} \quad (2)$$

4.4 BACTERIAL CELLULOSE ANALYSIS

This section was discussed about the chemical and physical characteristic of bacterial cellulose. The bonding in bacterial cellulose was determined by using Fourier Transform Infrared (FTIR) while the morphological structure of bacterial cellulose was determined by using Scanning Electron Microscope (SEM).

4.4.1 Fourier Transform Infrared (FTIR) Analysis

FTIR was used in order to determine the bonding in the bacterial cellulose based on the absorption of radioactive by the compounds chemical bonds. Figure 4.8(a) showed the spectrum of bacterial cellulose with 55.56% match to the cellophane spectrum. According to Elvie E.Brown and Marie-Pierre (2007), match percentage above 40% can be accepted as the main bonding in a sample. C-OH bonding (Oh et al., 2005), anti-symmetric bridge stretching of C–O–C (Kacurakova et al., 2002), H bond in OH group, aliphatic OH group (Moharram and Mahmoud, 2008), C-O stretching at C_3, C-O stretching and C-C stretching at C_6 (Oh et al., 2008) were the chemical bonding that present in cellulose molecular structure.



	Index	Match	Compound Name	Library Name
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2	290	45.37	Formaldehyde - aqueous solution	HR Nicolet Sampler Library
3	8	45.18	CELLOPHANE	Hummel Polymer Sample Library
4	335	44.72	Glycerol	HR Nicolet Sampler Library
5	14	43.94	METHYL ALCOHOL, 99.9%, SPECTROPHOTOM	Aldrich Vapor Phase Sample Library
6	109	40.19	SUCROSE IN KBR	Georgia State Crime Lab Sample Library
7	119	38.07	INOSITOL POWDER IN KBR	Georgia State Crime Lab Sample Library
8	1	37.53	Wed May 11 14:50:58 2005 (GMT+08:00)	KUKTEM2

Figure 4.8(a): Bacterial cellulose Spectrum

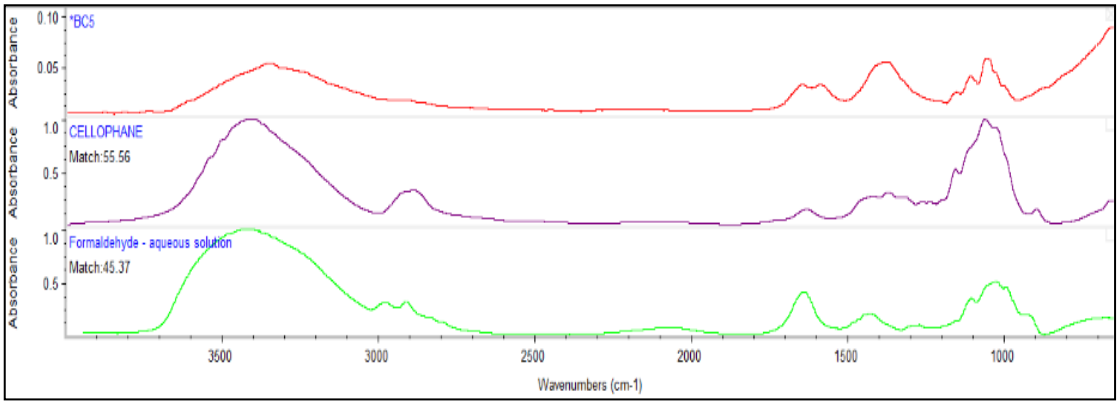

Figure 4.8(b): Comparison between bacterial cellulose and cellophane spectrum

Based on the result from FTIR analysis, Figure 4.8(a) showed the absorbance peak at 3337.69 cm^{-1}, 1377.27 cm^{-1} and 1056.42 cm^{-1}. According to Moharram and Mahmud (2008), the wavenumber for hydroxyl functional group was in range from 3256 cm^{-1} to 3853 cm^{-1}.Thus, wave number 3337.69 cm^{-1} in Figure 4.8(a) indicated the hydroxyl functional group (OH) of the bacterial cellulose. Besides that, the wavenumber 1377.27 cm^{-1} belongs to the bonding of carbon and hydrogen atom (CH) that appeared

in the bacterial cellulose structure. This was based on studied from Muenduen Phisalaphong et al.,(2008) which reported that wavenumber between 1300 cm^{-1} to 1400 cm^{-1} is the wavenumber for CH bonding. The wavenumber at 1056.42 cm^{-1} indicated the bonding of carbon and oxygen atom (CO) and it was proven by previous studied by Housni El-Said et al.,(2008) which stated the range from 1046 cm^{-1} to 1067 cm^{-1} was for C-O bonding. Therefore, it can be concluded that the product from the fermentation was consisted of mainly cellulose functional group. Table 4.7 below summarized the type of bonding for Figure 4.8(a).

Table 4.7: Summarized table

Wavenumber (cm^{-1})	**Type of Bonding**	**Reference**
3337.69	(O-H)	Moharram and Mahmoud(2008)
1377.27	(CH)	Muenduen et al.,2008
1056.42	(C-O)	Housni El-Said et al.,2008

4.4.2 Scanning Electron Microscope (SEM) Analysis

The morphological structure of bacterial cellulose that had been produced in this study was observed by using Scanning Electron Microscope. M.Fujita et al., 2002 studied about structure of bacterial cellulose and stated that bacterial cellulose chemical structure was similar with plant cellulose but the degree of polymerization differs from about 13000 to 14000 for plant and 2000 to 6000 for bacterial cellulose. Klemn et al.(2006) had mentioned that plant cellulose bound with lignin, hemicelluloses and other chemicals but bacterial cellulose was not bound to other chemicals and relatively high purity. Figure 4.9.1 and Figure 4.9.2 showed the bacterial cellulose structure for treated and untreated under Scanning Electron Microscope (SEM).

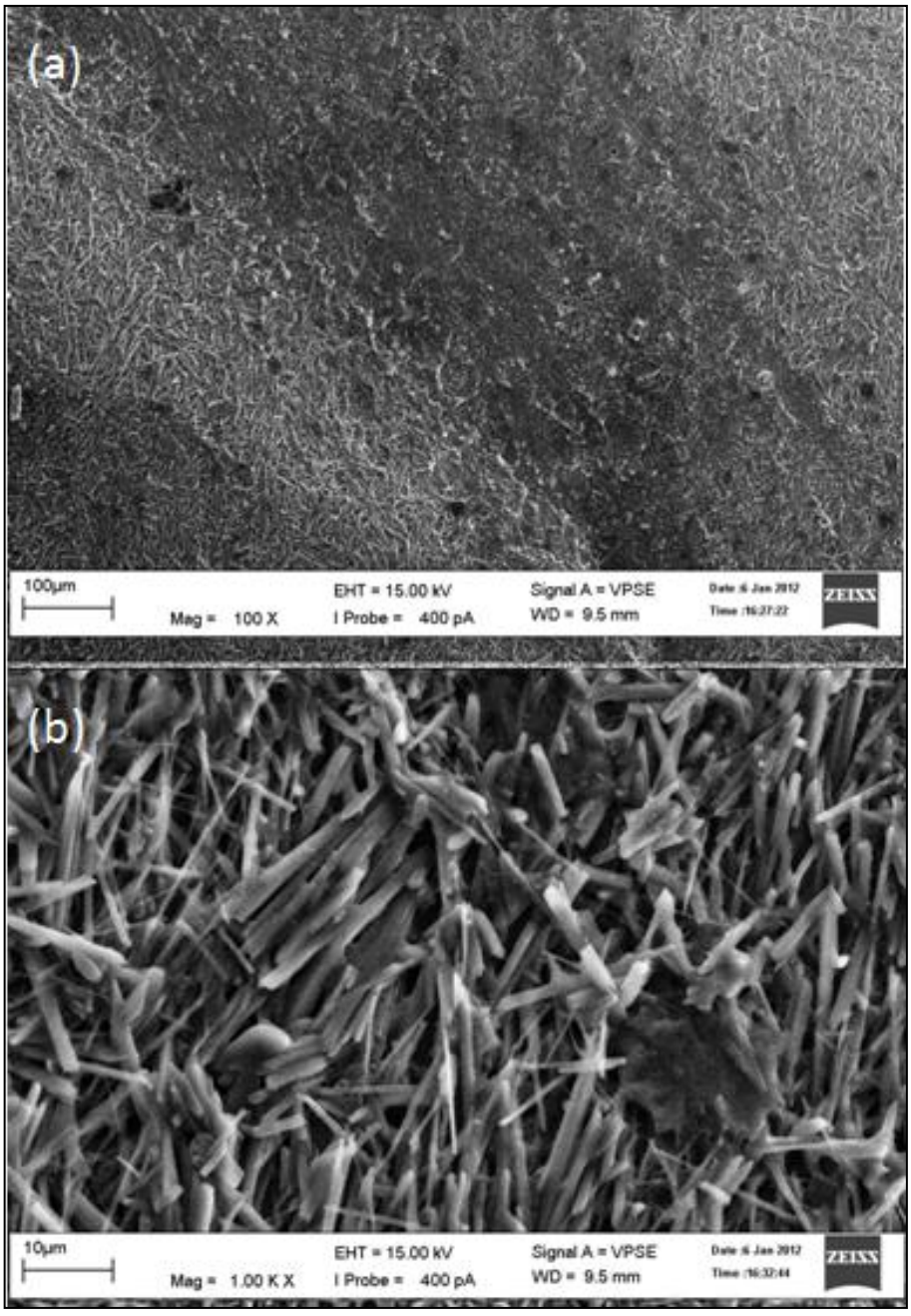

Figure 4.9.1: Surface structure of treated BC at (a) 100X (b) 1000X magnification

Figure 4.9.1 showed the structure of the treated bacterial cellulose. The treated BC pellicle was immersed in 1% sodium hydroxide (NaOH) solution for 1 day while the untreated BC pellicle was left on the sieve to allow the liquid from the pellicle to escape through the sieve without any treatment. Figure 4.9.1(a) was observed under 100X magnification. The microfibrils can be seen clearly on the surface of the BC film because the by-product and other impurities from cellulose have been removed by the

NaOH solution. S. Bielecki et al. (2005) stated that, by removing the non-cellulose materials such as protein and nucleic acid from bacterial cells, it allowed the formation of strong hydrogen bond between the microfibrils of the bacterial cellulose. Figure 4.9.1(b) at 1000X magnification showed clearly the structure of BC microfibrils. The microfibrils were the basic structure of bacterial cellulose which composed of glucan chain interlocked with hydrogen bond (Prashant R.Chawla et al.,2008).

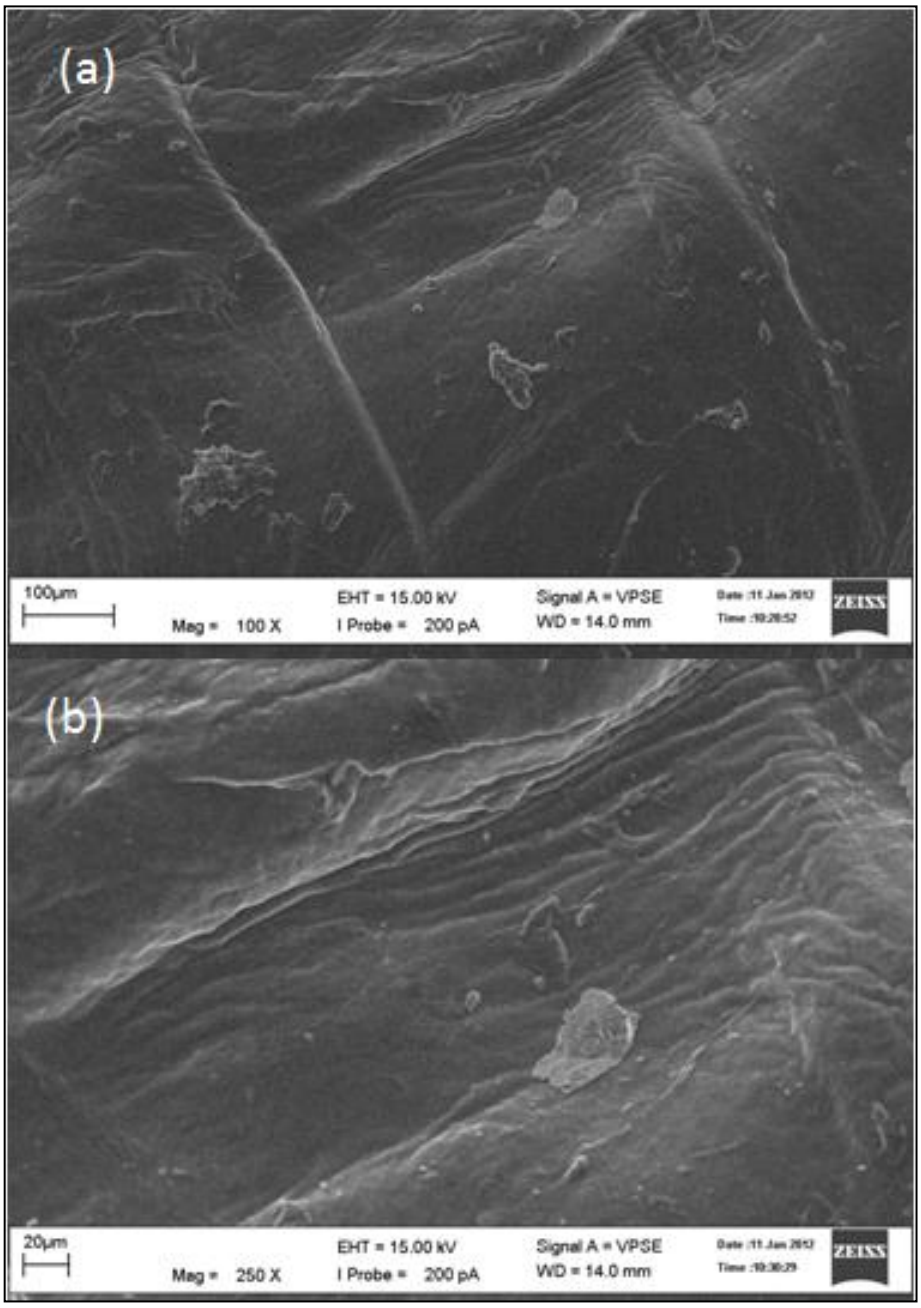

Figure 4.9.2: Surface structure of untreated BC at (a) 100X (b) 250X magnification

Figure 4.9.2 showed the surface structure of untreated bacterial cellulose. The different between treated and untreated BC was the purity of the bacterial cellulose itself. Treated BC has high purity since the impurities on the surface have been removed by NaOH while untreated BC was not pure because the impurities on the surface were not removed. The impurities, cellulose by-product and other organic compounds such as nucleic acid and protein remaining from the culture medium were trapped on the surface of the untreated BC. Thus, when the untreated BC was observed under SEM, the microfibrils on the surface cannot be seen because it was disturbed by the trapped impurities on the surface.

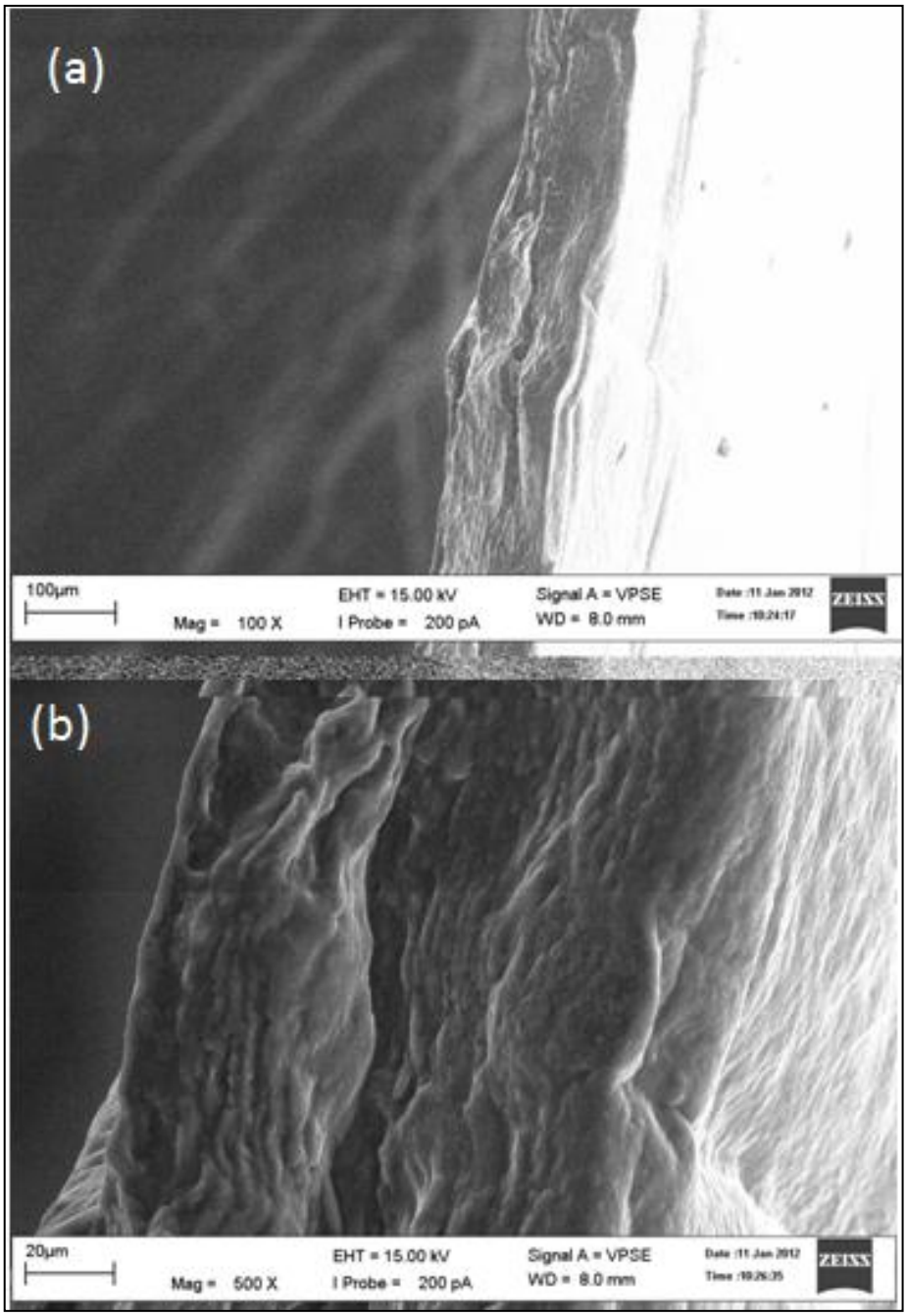

Figure 4.9.3: Cross section structure of bacterial cellulose at (a) 100X (b) 500X magnification

Figure 4.9.3 showed the cross section structure of bacterial cellulose under 100X and 500X magnification. The figures showed a dense structure of bacterial cellulose which formed by the strong intra and inter-fibrils hydrogen bond of BC. S.Bielecki et al. (2005) reported that intermolecular and intra molecular hydrogen bonds initially occur

in each cellulose sheet, and then the cellulose crystalline structure was formed with the development of hydrogen bonds between cellulose sheets. At 500X magnification it showed clearly the compactness structure of the bacterial cellulose. This compactness structure was reflected on the mechanical strength of the bacterial cellulose, where the Young Modulus of the bacterial cellulose with denser fiber networks was greater as compared with the less one. The mechanical strength of the bacterial cellulose membrane was also claimed depend on the quantity of the hydrogen bond formed in the bacterial cellulose (C.Tokoh et al.,2002). The high number of hydrogen bonds formed caused to more formation of fiber networks which has high porosity inside the bacterial cellulose film. Thus, the bacterial cellulose that produced with great thickness will exhibit a denser structure networks and performed a good quality of mechanical strength since it contained more hydrogen bond across the membrane thickness. These characteristics of bacterial cellulose leads to various applications in industry such as textile, medical, waste treatment and others.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

As the conclusion this study was about the optimization of bacterial cellulose production in the apple juice medium by using Response Surface Methodology (RSM). The variables that need to optimize were pH, temperature of cultivation and medium concentration. Each variable has been optimized by using RSM. A highly significant quadratic polynomial obtained by Central Composite Design (CCD). The optimum condition was 5.95, 95% and 30.3°C for pH, medium concentration and temperature respectively which able to produce 1.20g of dry bacterial cellulose. Meanwhile, the chemical and physical properties of bacterial cellulose also have been confirmed throughout this study by using Fourier Transform Infrared (FTIR) and Scanning Electron Microscope (SEM). The main functional groups in bacterial cellulose have been successfully determined by using FTIR while the morphological structure for surface and cross section of BC has been observed by using the SEM.

5.2 RECOMMENDATION

For this research improvement in future, there are some recommendations related to this study. Firstly, different strains use for the bacterial cellulose production cause to different yield of bacterial cellulose. By using another type of strains such as *Agrobacterium,Pseudomonas,Salmonella* is believed to produce bacterial cellulose with an acceptable yield. Secondly, there are some other factors or variables need to be taken into consideration for the bacterial cellulose production such as incubation time,

dissolved oxygen (DO) and others. These factors are believed to affect the bacterial cellulose production.

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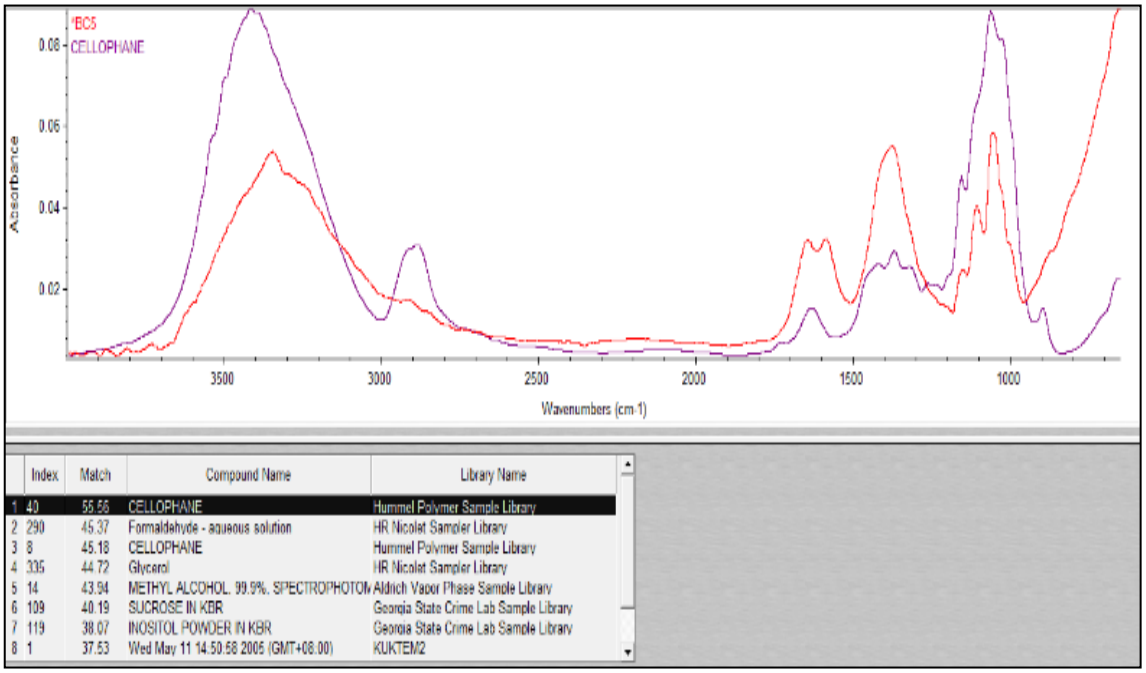
APPENDIX A

FOURIER TRANSFORM INFRARED (FTIR) SPECTRUM



	Index	Match	Compound Name	Library Name
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5	14	43.94	METHYL ALCOHOL, 99.9%, SPECTROPHOTOM	Aldrich Vapor Phase Sample Library
6	109	40.19	SUCROSE IN KBR	Georgia State Crime Lab Sample Library
7	119	38.07	INOSITOL POWDER IN KBR	Georgia State Crime Lab Sample Library
8	1	37.53	Wed May 11 14:50:58 2005 (GMT+08:00)	KUKTEM2

Figure A1: Overlay of cellulose and cellophane FTIR spectrum

APPENDIX B
CALCULATION

Sodium Hydroxide Dilution:

m = (MV/1000) x density of NaOH

= ((1M)(250mL)/1000) x 40 g/mol

= 10 g

Percentage of error:

$$\text{Percentage of error}(\%) = \frac{\text{Actual yield} - \text{Predicted Yield}}{\text{Predicted Yield}}$$

1) [(1.2173-1.20353)/1.20353] X 100% = 1.14%
2) [(1.3065-1.20332)/1.20332] X 100% = 8.57%
3) [(1.1986-1.20237)/1.20237] X 100% = 0.31%

Moisture:

[(Wet weight-dry weight)/wet weight] X 100%

[(28.3467g-1.2173g)/28.3467] x 100% = 95.70%