OPTIMIZATION OF BACTERIAL CELLULOSE PRODUCTION FROM *ACETOBACTER XYLINUM* BY USING RESPONSE SURFACE METHODOLOGY (RSM)

JALEHA BINTI SATHAR

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SIGNATURE : .....
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I dedicate this entire work to my family especially to my beloved parents, whose patient, support and companionship have facilitated my study, and made my life enjoyable, to my grateful siblings. And not forgot to my sources of inspiration, Mdm Zatul Iffah as energetic supervisor and to all my friends for their enduring faith and unconditional love in good times and bad.

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Last but not least, there will be errors, inconsistencies and over-simplifications in this thesis and I bear absolute responsibility for the erratic judgments I made. None of the above mentioned people should be held responsible for any errors in this thesis.

ABSTRACT

Nowadays, the application of cellulose as renewable polymer and bio materials has been a great attraction for most researchers to optimize the production of cellulose through the fermentation of *Acetobacter Xylinum* sp. The latest technology of the optimization process which known as Response Surface Methodology (RSM) was applied to determine the significant variables that affect the fermentation of bacterial cellulose. The variables were selected as temperature, pH and glucose concentration of the medium. Prior RSM, OFAT was conducted to determine the minimum range for each variable. The minimum range of temperature was selected at 25, 27, 29, 31 and 33 °C. pH was at 4,5,6,7 and 8, meanwhile glucose concentration was at 0, 2, 4, 6, 8 and 10 g/L. The range of optimum value for each variable determined from OFAT was inserted into RSM for further optimization. From the statistical analysis of RSM, all the three variables proved to significantly affect the fermentation process by probability value less than 0.05. Further optimization of the fermentation by using temperature at 29.2 °C, pH at 5.83 and glucose concentration at 1.75 g/L was enhanced the yield of bacterial cellulose at 3.6 times than the conventional fermentation condition, where 17.81 g of bacterial cellulose was determined after the optimization process. The typical spectrum of cellulose which consists of C-O ether bond, hydroxyl bond and C-H bond was successfully determined from the bacterial cellulose sample at wavenumber of 3331, 2920, 1500–1300 and 1025 cm^{-1} respectively from FTIR analysis. Meanwhile, the observation by SEM on the treated and untreated bacterial cellulose showed different observation of fibre network, where the treated bacterial cellulose showed clear fibre network as compared to the untreated sample. As the conclusion, the objective of the study was accomplished as the yields of bacterial cellulose were optimized 3.6 higher as compared to conventional method and the variables of pH, temperature and glucose concentration was proved significantly affected the bacterial cellulose fermentation process. Further investigation on other variables that affecting the fermentation process such as nitrogen sources concentration and cultivation technique by using other Box Behken and Plucket Burman as the optimization tools are suggested in order to analyze the benefits of the optimization tools provided by Design of Experiment (DOE).

ABSTRAK

Pada masakini, pengunaan selulosa sebagai polimer yang boleh diperbaharui dan bahan bio telah menjadi daya tarikan yang besar bagi kebanyakan penyelidik untuk mengoptimumkan pengeluaran selulosa melalui laluan biosintesis melalui penapaian *Acetobacter xylinum* sp. Teknologi terkini proses pengoptimuman yang dikenali sebagai Kaedah Permukaan Response (RSM) telah digunakan untuk menentukan pemboleh ubah penting yang memberi kesan kepada penapaian selulosa bakteria dan untuk menentukan nilai tertentu yang optimum bagi setiap pemboleh ubah. Pembolehubah telah dipilih sebagai suhu, pH dan kepekatan glukosa sederhana. Sebelum RSM, OFAT telah dijalankan untuk menentukan pelbagai optimum untuk setiap pemboleh ubah. Julat suhu optimum telah dipilih pada 25, 27, 29, 31 dan 33 °C. pH telah dipilih pada 4,5,6,7 dan 8. Sementara itu, kepekatan glukosa telah dipilih pada 0, 2, 4, 6, 8 dan 10 g/L. Pelbagai nilai optimum untuk setiap pemboleh ubah ditentukan dari OFAT telah dikecilkan dan dimasukkan ke dalam RSM untuk proses pengoptimuman lanjut. Dari analisis statistik RSM, ketiga-tiga pembolehubah telah dibuktikan dengan ketara memberi kesan terhadap proses penapaian dengan nilai kebarangkalian yang kurang daripada 0.05. Pengoptimuman lanjut penapaian dengan menggunakan suhu di 29.22 °C, pH pada 5.84 dan kepekatan glukosa pada 1.75 g/L telah dipertingkatkan hasil daripada selulosa bakteria pada 3.6 kali daripada keadaan penapaian konvensional, di mana 17.81 g selulosa bakteria telah dihasilkan selepas proses pengoptimuman. FTIR dan SEM telah dijalankan ke atas selulosa bakteria untuk mengkaji spektrum ikatan dan morfologi sampel. Spektrum tipikal selulosa yang terdiri daripada eter CO, CH dan hidroksil telah berjaya ditentukan daripada sampel selulosa bakteria pada 3331, 2920, 1500-1300 dan 1025 cm^{-1}. Sementara itu, perbezaan rangkaian fiber yang terdapat di dalam keratan rentas dan permukaan selulosa bakteria yang telah dicuci dan tidak dicuci telah digambarkan oleh SEM. Rangkaian fiber oleh keratan selulosa bakteria yang telah dicuci jelas kelihatan berbanding yang tidak dicuci. Oleh yang demikian, misi utama kajian ini telah tercapai setelah berat selulose bakteria tersebut telah dioptimumkan pada 3.6 ganda berbanding cara konvensional. Ini juga membuktikan bahawa faktor pH, suhu dan kandungan gula mempengaruhi fermentasi selulose bakteria dengan siknifikan seperti yang ditemui dalam kajian sebelum ini. Kajian lanjutan ke atas faktor lain yang mempengaruhi hasil selulosa bakteria seperti kepekatan sumber nitrogen dan kaedah penapaian dengan mengunakan kaedah pengoptimuman seperti Box Behken dan Plucket Burman adalah dicadangkan, dalam mempelajari kelebihan kaedah pengoptimuman yang disediakan oleh DOE.

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BC	Bacterial Cellulose
OFAT	One Factor At One Time
RSM	Response Surface Methodology
CCD	Central Composite Design
ANOVA	Analysis of Variances
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscope
g/L	Concentration
g	Gram
℃	Celsius
mL	Millimetre
%	Percent

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CHAPTER 1

INTRODUCTION

1.0 RESEARCH BACKGROUND

Production of bacterial cellulose through a fermentation process has a long history as the earlier production has begun since 1886 (Chawla et al., 2008). Since 18 centuries, researchers all over the world kept improving the fermentation process from many aspects including the cultivation technique, media components and other parameters that can enhance the bacterial cellulose production (Bielecki, 2005 and Panesar et al., 2009).

The study on bacterial cellulose production is receiving interest from industries since it gave huge significances through wide applications in various fields such as medicine, biopolymer, pulp and paper industry, food and tissue engineering (Bielecki, 2005; Keshk and Sameshima, 2005; Chawla et al., 2008 and Panesar et al., 2009).

The most application of bacterial cellulose is in the biopolymers production. Recently, biopolymer is preferably used compared to the petrochemicals derived polymers due to its biodegradable, biocompatible and environmental friendly to the nature. Reflecting on this, biopolymers is receiving great attention and becoming valuable in the market (Said et al., 2008).

In medical fields, it has been used to produce artificial skin, blood vessel, and scaffold for tissue engineering of cartilage and wound dressing. Special properties such as high water-holding capacity and porous structure makes the healing faster and

moisturizes the burns better than conventional process using gauze and ointments (Czaja et al., 2006). Moreover, researchers keep improving the characteristics of bacterial cellulose especially in producing high quality of wounds by addition of polyurethane, chitosan, starch and aloe vera (Saibuatong and Philasaphong, 2009). Further application in bone regenerations was successfully done by applying bacterial cellulose scaffolds with 300 until 500 µm of pore size into the bone cell of human. Results of this study indicate that the bacterial cellulose has been potential in producing 3-D scaffolds for bone graft productions in future (Zaborowska et al., 2010).

Furthermore, nowadays industry tends to replace the use of plant cellulose with bacterial cellulose. It is profoundly to highly purified and financially valuable for application in paper and textile industry. In comparison with the bacterial cellulose, plant cellulose needs further purification in order to remove the content of hemicellulose and lignin material (Said et al., 2008).

However, the production of bacterial cellulose in industrial scale was stunted due to the low productivity and time consuming. The productivity of the bacterial cellulose from the fermentation process was believed affected by several factors, such as temperature, pH and carbon sources. Through the optimization of these factors, researchers believed the yields of bacterial cellulose can be enhanced significantly.

1.1 PROBLEM STATEMENT

Cellulose has been used in various kinds of industry such as medical, food, artificial skin, textiles and paper industry. Most of the cellulose is taken from plant as the primary sources. Since the usage of plant cellulose in industries was resulted in pollution problem arisen from the pulping and purification problem, the replacement with bacterial cellulose has become one of the alternatives. Nevertheless, its production has low productiveness, which derived at high medium cost, where it takes account up to 60% of the total fermentation cost. Therefore, the application of coconut water as the fermentation medium can become as alternative due to its abundant in Malaysia and

worldwide. Although coconut water was widely used as the medium, the productivity of bacterial cellulose still remains low as the optimization of the bacterial cellulose fermentation using coconut water are inadequate. Hence, the productivity of bacterial cellulose from the fermentation process has a potential to be enhanced by the application of response surface methodology (RSM) where the control variable such as pH, temperature and glucose concentration that affected the fermentation process can be optimized. Thus, the optimization of bacterial cellulose production by the coconut water mediums can potentially reduce the cost and enhances the yields of bacterial cellulose significantly with the optimized conditions.

1.2 STATEMENT OF OBJECTIVE

The objective of this experiment is to optimize the bacterial cellulose production based on coconut water medium from *Acetobacter Xylinum* by using Response Surface Methodology (RSM).

1.3 RESEARCH SCOPES

1.3.1 The scope of the study is:

i. To investigate the optimum value of pH from the range of 4, 5, 6, 7 and 8.

ii. To investigate the optimum value of temperature from the range of 25, 27, 29, 31 and 33 °C.

iii. To investigate the optimum value of glucose concentration at 0, 2, 4, 6, 8 and 10 g/l.

iv. To study the interactions between 3 parameters (pH, temperature and concentration) during bacterial cellulose production process from the graphical of response surface plots.

v. To analysis the chemical and physical structure of bacterial cellulose by using FTIR and SEM method.

1.4 SIGNIFICANCE OF THE STUDY

A result of previous studies has indicated that the bacterial cellulose exhibited unique properties such as good mechanical strength, high porous structure, water holding capacity and biocompatible. In view of these results, the potential application of bacterial cellulose in various fields of industries such as food, medical, pulp and paper, textiles and biopolymer is widely open. Foreseeing its potential to be applied in various fields, its optimization in the fermentation process by using RSM has been chosen as the main interest in this study. With the optimized fermentation process, the yield of bacterial cellulose is believed to be enhanced significantly. Thus, it can substitute the use plant cellulose and avoid the forest depletion that can lead to the global warming.

CHAPTER 2

LITERATURE REVIEW

2.0 INTRODUCTION

The production of bacterial cellulose is a complex process which affected by multiple variables and required optimal conditions to achieve maximum production. A brief literature reviews about the production of bacterial cellulose and variables that affect the process will be discussed in this chapter.

2.1 BACTERIAL CELLULOSE

Cellulose is the most abundant compound on earth which 50 percent of the mass produced in plants through the photosynthesis process. The repetition straight chain of β-1,4 linked D-glucose units in cellulose is resulted as homopolymer compound and identified with a molecular formula of $(C_6H_{10}O_5)n$. Currently, cellulose is used as biopolymer in the attempt to replace petrochemicals derived polymer due to concern to the nature. Despite this, cellulose also has wide application in various fields such as in medical, food and textiles field. Apart from that, the most application of cellulose was reported in pulp and paper industry (Valjamae, 2002 and Ioelovich, 2008).

The research conducted by Keshk and Sameshima (2005) claimed that the usage of native cellulose origin from plants and trees leads to the environmental problems due to the removal process of hemicellulose and lignin material which contained in plant's cellulose. The pollutants resulted from the purification process had caused air, soil and water pollution as these wastes were not biodegradable and unable to decompose in

landfill. Moreover, separation and purification processes of plants cellulose required higher expenses (Cheng, 2009). In concern to nature and forest preservations, researchers decided to find an alternative ways to produce cellulose from others sources, which then they found the alternative source is from bacteria.

Previously, Chawla et al. (2009) claimed that cellulose can be produced in some gram-negative bacteria, such as *Acetobacter, Agrobacterium, Achromobacter, Aerobacter, Sarcina, Azotobacter, Rhizobium, Pseudomonas and Salmonella*. Since the cellulose is produced from bacteria, it is named as bacterial cellulose. Its molecular formula is same as the natural cellulose from plant, but differs in chemical and physical properties.

Works by Said et al. (2008) had shown the quality of bacterial cellulose was excellent than the plant cellulose when compared in terms of purity, degree of polymerization and crystallinity. Due to the reticulated of networks of fine fibres with diameter about 0.1 um, which was one hundredth than plant derived fibre., bacterial cellulose exhibited higher tensile strength and water holding capacity, which give credit for bacterial cellulose to be used as raw material for application in industries (Bielecki, 2005 and Chawla et al., 2008).

Foreseeing its potential in commercialization aspects, numerous studies are done in the attempt to improve the physical and chemical properties of bacterial cellulose by combining it with natural sources such as protein, aloe vera, betal leaves, chitosan, starch and gelatin. Works by Wiegand et al. (2006) had successfully enhanced the antioxidant properties of bacterial cellulose film through *in situ* fermentation of bacterial cellulose combined with collegen. Another research conducted by Saibuatong and Philasaphong (2009) had produced a nanostructure film that composed of bacterial cellulose and aloe vera. The film exhibited better tensile strength and reduced pore size compared to the unmodified bacterial cellulose film.

Further improvement on antimicrobial properties of bacterial cellulose was developed through the research conducted by Maneerung (2007). It was developed by impregnation of silver nanoparticles into bacterial cellulose via the adsorption process.

The results indicated strong antimicrobial activity of modified film against the gram positive and negative bacteria of *Escherichia coli* and *Staphylococcus aureus.*

Reflecting on this, the applications of bacterial cellulose in medical fields are receiving interest in pharmaceutical and medicines company. Starting from the usage as wound dressing, bacterial cellulose has been studied to be applied in replacing vascular tube and artificial skin. Researchers believed that bacterial cellulose has been potential to be sprout out into more critical applications such as surgical wounds, bedsores, ulcers, tissue and organ engineering in future (Fontana et al., 1990). Based on wide application and contribution of bacterial cellulose in various fields of industry, researchers believed that the continuous studies in bacterial cellulose production are significant and important where it can give benefit to human beings.

2.2 FERMENTATION OF BACTERIAL CELLULOSE

Bacterial cellulose was first found produced by the fermentation process developed by the resting cell of *Acetobacter,* with the supplement of oxygen and glucose (Chawla et al., 2008). Since that, researchers start hunting these alternative ways to produce bacterial cellulose in a bulk amount for wide applications in industry. Generally, factors affected bacterial cellulose yield from the fermentation process were reported as concentration of carbon sources, nitrogen sources, temperature and pH of the medium (Jagannath et al., 2008). In other hands, Sumate et al. (2005) had claimed that the cultivation technique also affected the quality and quantity of bacterial cellulose produced via fermentation process.

2.2.1 *Acetobacter Xylinum*

Acetobacter is believed to be the first bacteria producing bacterial cellulose in era 1886 through the studies conducted by Brown (1886). Several attempts had been made by previous researchers to study the production of bacterial cellulose by using bacteria strain. Gram-negative bacteria such as *Agrobacterium, Achromobacter, Aerobacter,Sarcina, Azotobacter, Rhizobium, Pseudomonas* and *Salmonella* are able to

produce cellulose (Saibuatong and Philasaphong, 2009). Cellulose is also synthesized by the gram-positive bacterium which is *Sarcina ventriculi*. The most effective producers of bacterial cellulose were reported as *Acetobacter xylinum, Acetobacter hansenii and Acetobacter pasteurianus* (Keshk, 2005 and Chawla et al., 2008).

Acetobacter is well known as the effective strain producing bacterial cellulose in higher yields compared to other bacteria. Exist as a rod-shaped and gram-negative bacterium, *Acetobacter* also able to grows and produces bacterial cellulose from a wide variety of substrates and is devoid of cellulase activity. This bacteria also exist with another name, which is *Gluconacetobacter xylinus* (Keshk and Sameshima, 2005 and Ross et al., 1991).

The effectiveness of this strain is due to the ability to produce bacterial cellulose with a high degree of polymerization from a wide range of carbon and nitrogen sources. It has been produced bacterial cellulose from various carbon sources such as glucose, fructose, sucrose and others. On the other hand, *Acetobacter* was reported to have efficiency of 50 % in converting hexoses, glycerol, dihydroxyacetone, pyruvate, and dicarboxylic acids into bacterial cellulose. Researcher also believed that *Acetobacter* is the suitable bacterial species for large scale bacterial cellulose production (Keshk et al., 2006). Thus, the selection of *Acetobacter xylinum* as bacteria strain for this study is relevant and ideal for the production of bacterial cellulose.

2.2.2 Medium for Fermentation of Bacterial Cellulose

Based on the study of bacterial cellulose fermentation process, Chawla et al. (2009) stated that the medium used in a fermentation process play an important role as the main sources of nutrients needed by *Acetobacter* strains to synthesis cellulose during the cultivation period. Basically, the nutrients required for the process are carbon, nitrogen, phosphorus, sulphur, potassium and magnesium salts. Additional supplement of amino acids and vitamins are also used to promote the cell growth and enhance the production of bacterial cellulose.

The conventional medium used for bacterial cellulose production is known as Schram Hastrin (SH) medium which had been introduced in era 1954. The SH medium contains glucose (20 g/L) as the carbon source, yeast extracts (5 g/L) , peptone (5g/L) , citric acid (2.7 g/L), disodium phosphate (1.17 g/L) (Panesar et al., 2009). The medium was successful in supplying the nutrients for bacterial cellulose synthesis using *Acetobacter* strains and were used frequently in previous studies. However, components in SH medium are expensive and thus become the limitation factor of large scale bacterial cellulose production in industry.

In the attempt to reduce the cost of medium, numerous types of byproduct media which mainly origins from agricultural waste were studied (Said et al., 2008). Results of previous studies indicated that agricultural wastes such as pineapple, empty palm oil fiber, corn steep liquor (CSL), beet molasses, sugarcane molasses and coconut water could support the growth of *Acetobacter* strains and able to produce bacterial cellulose significantly (Said et al., 2008; Retegi et al., 2009 and Keshk et al., 2006).

Studies conducted by Said et al. (2008) had shown corn steep liquor (CSL) had been potential as the best waste medium for bacterial cellulose production. The cultivation of *Acetobacter* strain on CSL medium by using biofilm reactor for three days was resulted with yields about 7.05 g/L. The similar approach taken by Cheng (2009), where the studies on bacterial cellulose production by using CSL medium resulted in 4.695 g/L of bacterial cellulose gained after three days of static cultivation. Compared to other waste medium, researchers only obtained about 1.75 g/L to 2.82 g/L of bacterial cellulose.

Unfortunately, it’s unsuitable to use CSL medium in this study due to the limitation of sources and time. In other hand, Said et al. (2008) have reported the saccharification process of the agricultural waste need to be done before used in medium preparation. In some cases such as using palm oil fiber, several treatments need to be done to achieve suitable condition for making the medium. This makes the preparation process of mediums become more complex, takes time and required higher cost. Thus, the idea of using readily made mediums is the best choice for this study

since many repetitions of a fermentation process need to be run in Response Surface Methodology (RSM).

The readily made medium mentioned above is coconut water. The usage of coconut water as the medium for Nata De Coco and vinegar productions had been applied since long time ago. The capability of coconut water in producing Nata De Coco without any additional nutrients supplements and carbon sources is proven since the citizen of the rural area in India and Filipinos managed to produce the gelatinous form of bacterial cellulose through the simple fermentation process by using coconut water as the mediums. Furthermore, previous studies on fermentation of bacterial cellulose by using coconut water as the mediums were successively done and obtained a significant amount of bacterial cellulose (Saibuatong and Philasaphong, 2009, and Rika and Yudianti, 2008). Hence, the conditions of the bacterial cellulose fermentation using coconut water as the medium will be optimized through the application of Design Expert software in this study.

2.2.3 Cultivation Technique

Despite fermentation conditions such as pH, temperature and carbon sources concentration, the cultivation technique also affects the quality and quantity of bacterial cellulose production. Researches conducted by Suwannapiunt (2007) and Sumate (2005) had studied on the effect of cultivation technique on the production of bacterial cellulose by using static culture, shake culture and reactor. Based on the results, the amount of bacterial cellulose produced by agitated and reactor culture was higher than static culture.

However, the quality of bacterial cellulose produced by static culture is significantly better than others. Images of SEM taken on the surface of bacterial cellulose produced by static culture have shown stringent fibrils fabrication with huge cell size. In fact, bacterial cellulose produced by static culture has good capability of water-holding rather than bacterial cellulose produced from other cultivation techniques (Sumate, 2005). In other hands, the fibrils was arranged in good arrangement and making it suitable for parchment of paper (Suwannapinunt, 2007).

Previously, Lee et al. (1999) claimed that the aggregations formed during shake culture was accelerated by the bacterial cellulose and resulted in formation of low quality of pellicles and disorderly fibrils arrangement. Furthermore, Yang (1998) has reported study on the production of bacterial cellulose under agitated condition was resulted with culture instability, where the strain was unable to synthesize cellulose due to inability to convert glucose into gluconic acid. Moreover, the accretion of non-producing mutant cell during the cultivation under agitated conditions was inhibited the growth of cellulose-producing cell.

Further study on improving the cultivation of bacterial cellulose via continuous stir tank reactor (CSTR) with the application of a static cellulose microfibril attachment (SCMA) matrix located inside the reactor was conducted by Krusong et al. (2000). The highest yields produced from the study are only 5.94 dry Wt/L, which are comparable with the amount of bacterial cellulose produced by static culture and shake culture. Inspite of taking high cost, application of CSTR modified with SCMA in bacterial cellulose production required technical monitoring to ensure all procedure of conducting the reactor run smoothly. Excessive of oxygen supplied into the reactor affected the ability of *Acetobacter* to produce cellulose due to the direct oxidation in the medium. Hence, the selection of static cultivation method for this study is significant due to its benefits in producing high quality of bacterial cellulose with reasonable cost and requires less supervision during the cultivation period.

2.2.4 Cultivation Time

The other important aspect of a bacterial cellulose fermentation process is about the cultivation time taken for the fermentation process. Basically, the cultivation time reflected on the weight and yield of bacterial cellulose formed at the end of the fermentation process. Studies conducted by Saxena et al. (2000) and Jung et al. (1999) showed that the cultivations time are optimum around 48 hours to 72 hours of a fermentation process.

Figure 2.1 and 2.2 illustrated the relation between cultivation time and weight of cellulose produced during the fermentation process. In Figure 2.1, the weight of a

bacterial cellulose increase proportionally from zero hours until reach 48 hours and the production rate decrease when longer time taken (Saxena et al., 2000). In other hands, weight of bacterial cellulose was linearly increased until it reaches 72 hours of a fermentation process as showed in Figure 2.2 (Jung et al., 1999).

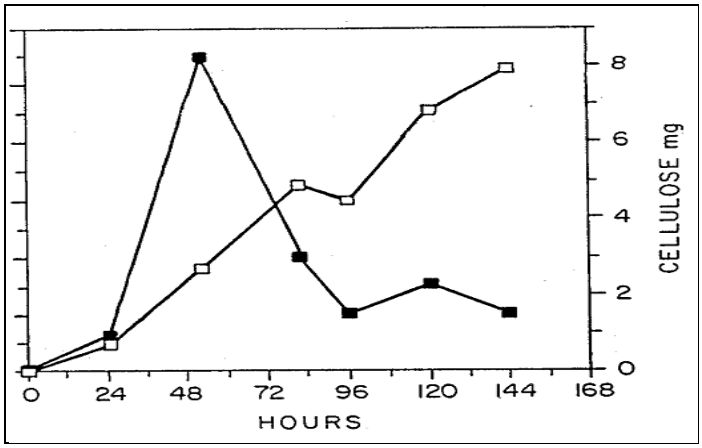

Figure 2.1: Cultivation Time of Bacterial Cellulose.

Source: Saxena et al. (2000)

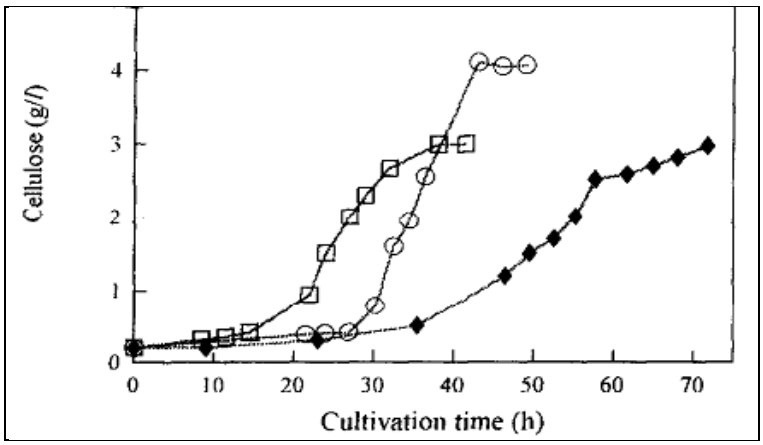

Figure 2.2: Cultivation Time of Bacterial Cellulose

Source: Jung et al. (1999)

Nevertheless, the cultivation time also reflected on the degree of polymerisation of bacterial cellulose produced from the fermentation process. Study conducted by Barbara et al. (2008) had revealed that the cultivation time more than 4 days was resulted with production of bacterial cellulose with a lower degree of polymerisation. As a renewable biopolymer, the degree of polymerisation of bacterial cellulose plays an important key to exhibit the properties of biopolymer (Klemm, 2007). Thus, longer time of cultivation is prevented to achieve a high quality of bacterial cellulose produced from the fermentation process.

Moreover, longer cultivation time of the fermentation process subsequently required high volume of medium and inoculum to support the production of bacterial cellulose during the cultivation period. Incubation at 360 hours which conducted by Marzieh and Ali (2010) was required 2000 ml of mediums to support the growth of *Acetobacter* strain in order to accomplish the bacterial cellulose production.

Recently, most of the researchers tend to use the short incubation time since the main objective of their research is to optimize the production of bacterial cellulose by investigating the significant variable that affects the fermentation process such as pH, temperature, broth ratio and medium concentration by using Design of Experiment (DOE). For example, the study on optimization conducted by Liang et al. (2010) was used two to three days only for the cultivation of the medium. Another study conducted by Hiroshi (1999) was used three days for the cultivation process. Since the cultivation time does not reflected on the optimization process significantly, the author believed the choices of three days for the cultivation time is relevant to the study.

2.3 EXPERIMENTAL DESIGN

2.3.1 One-Factor-AT-One-Time (OFAT)

OFAT is known as the conventional method in determining the effect of several variables on the fermentation process. The influences and behaviour of variables on the

process are studied by varying the level of one variable while keep the other variables constant. After finished investigating on one variable, the experiments proceed with the other variable until all variable is finished (Yannie, 2006). In this study, there are three variables studied by using OFAT method, which are pH, temperature and glucose concentration of the medium. Previously, the culture conditions such as temperature, pH and carbon source concentration was claimed as the crucial factors that affect the production of bacterial cellulose through fermentation process (Coban and Biyik, 2011). Therefore, numerous studies on these factors have been carried out in order to optimize the bacterial cellulose production.

Study of different pH and temperature for bacterial cellulose production in the Hestrin-Scharm (HS) mediums was successfully conducted by Coban and Biyik (2011). In the study, the researchers studied pH at range of 2.5, 3.5, 4.5, 6.5, 7.5 and 8.5, meanwhile the temperature was studied at range of 4, 22, 30 and 37 °C. Based on the results, the optimum yield of bacterial cellulose was observed at pH of 6.5 and temperature at 30 °C. In contrast, the study conducted by Jung (1999) and Verschuren (2000) has reported pH at 4 to 5 as the best range for production of bacterial cellulose. However, most of the researchers agreed with the range of temperature at 28 to 30 °C as the ideal temperature for production of bacterial cellulose.

On the other hand, the minimum range of optimum value for glucose concentration was studied by Keshk and Sameshima (2005). The best range for glucose concentration was observed at 1 to 1.5 %. Furthermore, most of the researchers tend to use about 2% of glucose concentration in preparing mediums for bacterial cellulose fermentation in their studies (Clasen et al., 2006).

The formation of by-products in the medium during the fermentation process has claimed as the main reason for the reduction of cellulose yield. The consumption of excess glucose concentration during the fermentation process has led to the formation of gluconic acid in the medium. Where when large amounts of gluconic acid present in the mediums, the mediums are turned into more acidic and yet decreased the pH of the medium. The decrease of medium's pH has affected the growth of *Acetobacter Xylinum* strains and reflected on the rate of cellulose production (Chawla et al., 2009).

2.3.2 Response Surface Methodology (RSM)

The main objective of this study is to find the optimal value for the three variables which are pH, temperature and glucose concentration in the medium. In preliminary studies on bacterial cellulose productions, researchers usually used one-factor-at-a-time (OFAT) to get the minimum range of optimal value for each variable. However, the traditional method of one factor at a time is no longer significant to be used in this study due to the limitations of time and incapable of determine the interactions among the variables. The alternative effect reflected from the various contents of the medium was easily misinterpreted regarding to the weakness of this method (Venkata et al., 2009 and Karunanithy and Muthukumarappan, 2010).

Nevertheless, the weakness of OFAT method is currently predominated with the application of Design of experiments (DOE). Recently, DOE are used in most of the optimization process which includes the optimization of the fermentation process (Nermeen et al., 2010). DOE was first developed by Sir Ronald A. Fisher at 1920s. Experimental design is a statistical method which generates a mathematical model to examine the relationship of variables that affecting a process and find out the responses of the process. This design software consists of five main category includes Response Surface Methodology (RSM) (Jinaphorn, 2009).

Yannie (2006) had reported that Response Surface Methodology was one of the statistical methods which were able to optimize the production in various fields such as food, biotechnology processes and biomass pre-treatment. RSM has been applied to optimize culture medium and other process variables for production of Tannase, Lipase and other enzymes. With fewer experiment trials, RSM is capable to identify many variables and their interactions during the production process (He and Tan, 2006 and Hanrahan et al., 2007).

RSM worked upon the optimization process through statistical analysis of the data collected from the experiments. The interpretation of the experiment's data by RSM then resulted in the form of regression equation. The regression equations

generated by analysis of variances (ANOVA) in RSM software represent the relation between the yield and the variables.

At the end of analysis by RSM, there are some images of graphical curvature is given, which is illustrated based on the regression equations generated by ANOVA, which represent the response surface of the interaction occurred between the variables toward the yield of the fermentation process (Jinaphorn, 2009). The significance of the optimized fermentation process by RSM is evaluated based on the fit of the regression equation and statistical analysis.

The effectiveness of regression equation is depended on the value of sum of squares (R^2). Meanwhile, the fit of statistical analysis is depended on the value of F test. The other important information of the statistical analysis is listed as a value of lack fit and *p*-test. The desired value of R^2 was reported in the range of 80 to 99 %. R^2 value is an important thing in analyzing the results of RSM, because it showed the ability of the model designed by the software in optimizing the fermentation process. High value of R^2 and F value shows the experiment model designed by RSM is effective in optimizing the fermentation process and has good variability in the responses of the experiment model (Jinaphorn, 2009 and Karunanithy, 2011).

In other hands, the level of interaction between the variables is evaluated based on the *p* value. The *p* value also indicated the influence of variables on the productivity of the process. Small value of *p*-test indicated a good experiment model (Venkata et al., 2009). Table 2.1 shows an example of ANOVA analysis results, where the value of R^2 at 98.14 in the table showed that the model is fit and able to explain almost all the responses on the process.

Table 2.1: Analysis of Variances (ANOVA)

Source of variation	Sum of squares	Degrees of freedom	Mean square	F-value	*p*-value
$X_1 + X_1X_1$	80.7	2	40.4	52.9	0.000001
$X_2 + X_2X_2$	220.9	2	110.5	144.7	0.000000
$X_3 + X_3X_3$	94.8	2	47.4	62.1	0.000000
$X_4 + X_4X_4$	109.5	2	54.8	71.7	0.000000
X_1X_2	3.3	1	3.3	4.3	0.059910
X_1X_3	16.0	1	16.0	20.9	0.000635
X_1X_4	4.6	1	4.6	6.05	0.030000
X_2X_3	14.1	1	14.1	18.42	0.001047
X_2X_4	3.7	1	3.7	4.85	0.047862
X_3X_4	11.2	1	11.2	14.7	0.002378
Error	9.2	12	0.76		
Total SS	492.9	26			
$R^2 = 0.9814$					

Source: Venkata et al. (2009)

2.4 ANALYSIS OF BACTERIAL CELLULOSE.

Generally, bacterial cellulose produced from the fermentation process is analyzed to find out the morphology, chemical and physical properties of the bacterial cellulose. According to Klemm (2007), cellulose morphology was observed as well organized fibrillar elements which own diameter about 3.5 nm. Meanwhile, physical and chemical characteristic of cellulose was illustrated as a high crystalline structure which constructed strongly by hydrogen bond and Van der Waals forces. Reflecting on this, cellulose is exhibit waterproof properties (Spiridon et. al., 2010).

Instead of chemical properties, researchers are more interested in investigating the morphology and physical properties of bacterial cellulose. Researchers have revealed that the chemical composition of bacterial cellulose is similar to plant cellulose and has no influence even after various changes are introduced in a fermentation process. In fact, its morphology is hooked with their mechanical and physical properties. Therefore, the analysis of bacterial cellulose in this study is only focused on the morphology and physical properties by conducting FTIR and SEM methods.

2.4.1 Fourier Transform Infrared (FTIR)

The analysis of FTIR is mainly conducted to investigate whether the peak of cellulose material does exist or not in the samples produced in the study. Numerous analyses of chemical structure and various macromolecules contained in bacterial cellulose was carried out by using FTIR methods. The absorption at certain wavelength indicates the type of macromolecules detected. The typical spectrum of cellulose consisted of hydroxyl, aliphatic C-H stretch, bending C-H stretch and C-O ether bonds (Klemm et al., 2001 and Vazquez et. al., 2006).

Table 2.2 listed the typical bond existed in cellose and the wavenumber of the bonds. The hydroxyl groups represented by the wavenumber of 3330 cm^{-1} and the aliphatic bond are represented by 2920 cm^{-1}. Meanwhile the bending C-H stretch bonds represented by wavenymber ranged from 1300 to 1500 cm^{-1}. The C-O ether bond represent by 1025 cm^{-1}. The FTIR spectrum of cellulose is illustrated by Figure 2.3.

Table 2.2: Typical Bond Existed in Cellulose Spectrum

No.	Bond	Wavenumber (cm^{-1})
1.	Hydroxyl groups.	3330
2.	Aliphatic C-H stretch.	2920
3.	Bending C-H stretch.	1300-1500
4.	C-O ether bonds.	1025

Source: Vazquez et al. (2006)

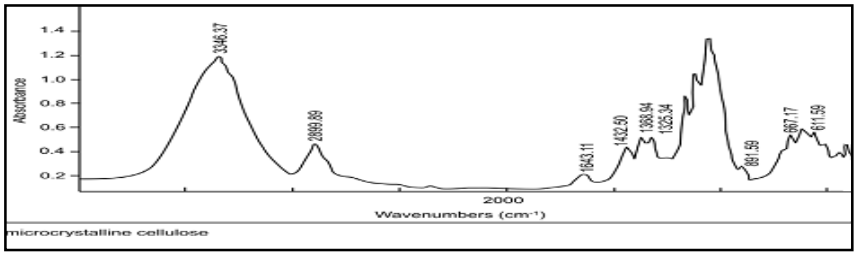

Figure 2.3: FTIR Spectrum of Cellulose

Source: Goh et al. (2012)

2.4.2 Scanning Electron Microscopy (SEM)

Microscopic study by using SEM were frequently done by researchers to observe the network structure of bacterial cellulose nanofibres, the surface of the fibres, the organization of fibre layer and the cell density in the bacterial cellulose (Lee, 1999 and Hernane, 2011).

Figure 2.4 shows some examples of SEM analysis on bacterial cellulose produced in previous studies. The figure shows the fibre network contained in the cellulose. The fibre structure of fibre is well organized and has porous structure. Based on previous studies, the dehydrated bacterial cellulose was cut and covered with gold. The observations were done on the emission field at acceleration voltage of 10 to 15 kV and the pictures were taken at 500x magnification (Hernane, 2011).

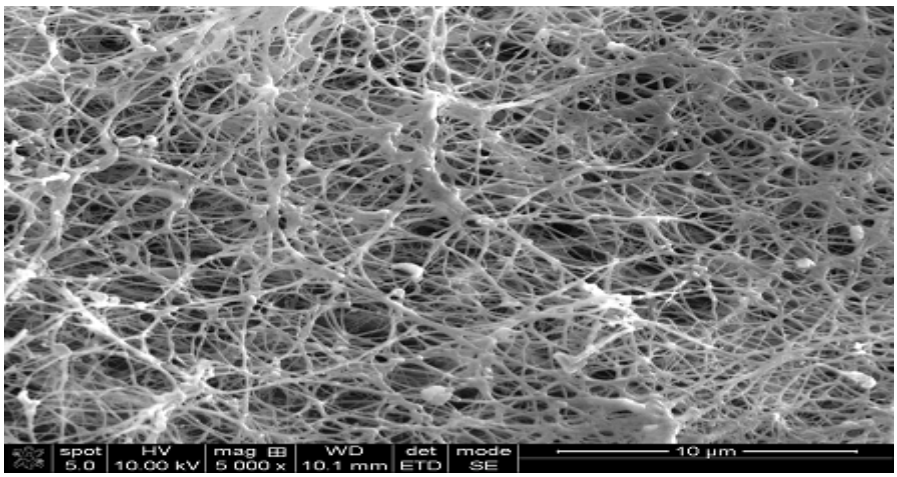

Figure 2.4: SEM Images of Bacterial Cellulose

Source: Hernane (2011)

According to Gea et al. (2011), the treatment of bacterial cellulose with sodium hydroxide solutions (NaOH) affects the observation of morphology through SEM analysis due to the impurities resulted from the fermentation process covered the surface of the bacterial cellulose. The impurities inhibit the better observation on the fibre network of the bacterial cellulose. The two step treatment of NaOH and NaCI was

proposed in her study, where the two treatments purified the bacterial cellulose better than the single treatment with NaOH.

The other main application of SEM in the studies of bacterial cellulose is to observe the changes on the fibre networks after certain modification was made in preparing the composites which consist of certain additional material such as silver nanoparticles, starch, aloe vera and collegen. The high porous structure and the biodegradability of bacterial cellulose fiber network makes it as suitable candidate to be apply in producing polymer nanocomposites and potentially can be applied in medical fields (Maria et al., 2010).

Nevertheless, the studies of bacterial cellulose production based on different type of medium also required SEM analysis in analyzing the differences of fibres networks produced from each type of medium. From the review, the structure of fibres produced from fruits juice medium relatively same with the fibre produced from commercial cellulose membrane. The differences were observed from the density of the fibre network and their porosity (Goh et al., 2012; Retegi et al., 2010).

Same observation of SEM analysis was resulted in the studies of different cultivation technique of bacterial cellulose. The fibre network formed from the static and shaking fermentation of bacterial cellulose was differentiated from the density and the porosity aspects (Cai and Kim, 2010 and Czaja et al., 2004).

From the review, the treatment aspect contributed mostly on the fibre network observation since it controlled the observation level on the fibre network. Thus, the author decided to investigate the treatment effect based on the bacterial cellulose produced in the present study by comparing the observation on the treated and untreated sample.

CHAPTER 3

METHODOLOGY

3.0 INTRODUCTION

The third chapter of the thesis is about the procedures and methods used in the study of optimization of bacterial cellulose production by using RSM. The study begins by conducting OFAT experiments and continued by an optimization process through application of RSM. The study is finalized by the production of bacterial cellulose by using optimization conditions which proposed by RSM. The optimized bacterial cellulose produced will be characterized using FTIR and SEM in order to analyze the morphology and physical structures of the bacterial cellulose.

3.1 MATERIALS AND SOLVENTS

The main material used in this study was *Acetobacter Xylinum* which was purchased from Malaysia Agricultural Research and Development Institute, Serdang, Selangor. Another material was coconut water, which purchased from the market. Meanwhile, other chemicals such as Ammonium Sulphate (NHSO4), Sodium Hydroxide (NaOH), Acetic Acid, distilled water and Glucose are purchased from Merck.

3.2 METHODS AND EXPERIMENTAL DESIGN

The overall procedure of the study is concluded in Figure 3.1. OFAT is conducted to find the range of optimum value for three variables, which are glucose concentration, pH and temperature of the fermentation process. Then, the range resulted from OFAT is inserted into RSM to detect the significance variables toward the production process of bacterial cellulose.

The optimized fermentation process is validated by conducting the fermentation process based on the optimum value of each variable proposed by RSM. The yield of bacterial cellulose produced from the optimized process is compared with previous studies to observe the difference. The sample is further analysed by using FTIR and SEM to investigate the existence of cellulose material on the sample produced from the fermentation process (Yannie, 2006).

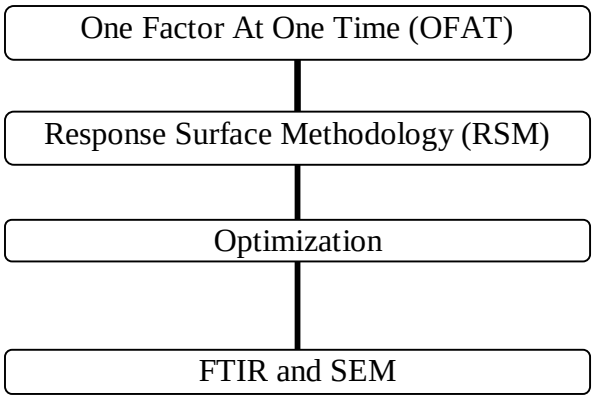

Figure 3.1: Flow Diagram of Procedures

Source: Yannie (2006)

3.2.1 Fermentation of Bacterial Cellulose

The static fermentation of bacterial cellulose was carried out inside a 250 ml conical flask, which containing 100 ml of medium and 10 ml of stock culture. The medium used in this study was coconut water and some additional chemicals. The

fermentation medium was incubated at various temperatures, pH and glucose concentration of medium. A further explanation was provided in next subtopic.

The fermentations process was conducted for 3 days by put the conical flask inside the incubator, in order to get required temperature for each set. The fermentation medium was made by mixing 100 ml coconut water, 8 g of glucose and 0.5 g of ammonium sulphate. The stock culture was made by mixing 100 ml coconut water, 8.0 g glucose, 0.5 g ammonium sulphate, a few drops of acetic acid and 10 ml *Acetobacter Xylinum* strains (Iffah, 2006).

3.2.2 One-Factor-At-One-Time (OFAT)

Three runs were set up to find the minimum range of optimum value for the variables that affecting the fermentation of bacterial cellulose, which are temperature, pH and glucose concentration.

3.2.2.1 Temperature

The bacterial cellulose fermentation process was conducted at constant value of pH and glucose concentration, in order to find the minimum range of optimum value for temperature that produces the optimum yield of bacterial cellulose. The fermentation was run at temperature of 25, 27, 29, 31 and 33°C. The temperature was controlled by manipulating the settings of the incubator, which used to provide the required temperature for the fermentation process (Keshk and Sameshima, 2005 and Pourramezan et al., 2009).

3.2.2.2 pH

The study was conducted at pH range of 4,5,6,7 and 8 in order to find the minimum range of optimum value for pH. The temperature and glucose concentration was constant at 30 °C and 2 g/L during the study of pH. The pH of the medium was adjusted by manipulating the amount of NaOH and Acetic acid used to prepare the

medium. NaOH was used to increase the pH and Acetic acid was used to decrease the pH (Jonas and Farah, 1998; and Panesar et al., 2009).

3.2.2.3 Glucose Concentration of the medium

The glucose concentration of the medium was studied at range of 0, 2, 4, 6, 8 and 10 g/l respectively. At the end of OFAT experiments, three graphs was plotted to analysis the results. The Y-axis represented the yield of bacterial cellulose meanwhile, the X-axis represented the variables. The range of optimum values was taken from the highest peak of the graph. These ranges of optimum values were used in RSM to get the specific value for each variable and the interactions among the variables (Keshk and Sameshima, 2005 and Pourramezan et al., 2009).

3.2.3 Response Surface Methodology (RSM)

The experiment was continued by using RSM to get the specific optimum value for each variable which was temperature, pH and glucose concentration of the medium. The standard type of RSM was Central Composite Design (CCD) which was used to optimize three types of variables of fermentation process (Yannie, 2006 and Karunanithy and Muthukumarappan, 2011).

3.2.3.1 Central Composite Design (CCD)

The minimum range of optimum value resulted from OFAT experiments were used to design the experimental model by CCD. The experimental design was consisted of 20 runs of experiments which consider each effect of variables. Each run suggested by CCD has different value of variables. The experimental model is showed in Table 3.1. The data resulted from each run was analyzed by analysis of variances (ANOVA).

The evaluation of ANOVA results was made based on the sum of square value (R^2), *p* value and F test (Liang et al., 2010). The optimal value for each variable was observed through the curvature of the 3-D plot. The optimal value of each variable was applied in the final fermentation process of bacterial cellulose. The yield of bacterial

cellulose produced from the optimized process was compared with previous results of other researchers to obtain the percentage differentiation (Nermeen et al., 2010).

Table 3.1: Experimental Model Designed by CCD

Run	Factor A: Glucose	Factor B: pH	Factor C: Temperature	Yields (g)
1	2.00	6.00	30.68	
2	2.00	6.00	29.00	
3	2.00	6.00	29.00	
4	3.00	7.00	28.00	
5	1.00	5.00	30.00	
6	0.32	6.00	29.00	
7	3.00	7.00	30.00	
8	1.00	7.00	30.00	
9	2.00	6.00	29.00	
10	2.00	6.00	29.00	
11	2.00	4.32	29.00	
12	3.00	5.00	28.00	
13	2.00	6.00	29.00	
14	2.00	6.00	29.00	
15	2.00	7.68	29.00	
16	1.00	7.00	28.00	
17	1.00	5.00	28.00	
18	3.00	5.00	30.00	
19	2.00	6.00	27.32	
20	3.68	6.00	29.00	

3.2.4 FTIR Analysis

FTIR spectrophotometer was used to identify the chemical bonds and specific functional group of the membrane. The FTIR spectrum of the bacterial cellulose was measured in the wavelength range from 500 to 4000 cm^{-1}. Spectral output was recorded in absorbance as a function of wavenumber (Saibuatong and Phisalaphong, 2010). The FTIR Nicolet Avatar 370 DTGS was used in the study. The images of FTIR spectrophotometer was attached in Appendix A.

3.2.5 Scanning Electron Microscopy (SEM)

SEM was used to generate high resolution images of surface and cross section of bacterial cellulose. SEM was used at an acceleration voltage of 15kV. The bacterial cellulose was frozen in liquid nitrogen. The sample was snapped by vacuum dried and then sputtered with gold (Saibuatong and Phisalaphong, 2010). The SEM analysis was performed using SEM EDX Spectrometer EVO 50. The images of SEM equipment was attached in Appendix B.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.0 INTRODUCTION

This chapter cover the results and discussion from the two stages of optimization process on bacterial cellulose fermentation process, which are One Factor at A Time (OFAT) and Response Surface Methodology (RSM). The minimum range of optimum value for glucose concentration, pH and temperature of bacterial cellulose fermentation process were studied by using conventional method of OFAT. The minimum range of optimum value for the variables was then optimized by using RSM to obtain the optimum yield of bacterial cellulose from fermentation process. The morphology and chemical properties of bacterial cellulose produced from the optimized fermentation process were analysed by using FTIR and SEM.

4.1 ONE FACTOR AT ONE TIME (OFAT)

4.1.1 Minimum Range of Optimum Value for Glucose Concentration

The optimum value for glucose concentration was studied at 0, 2, 4, 6, 8 and 10 % of glucose concentration in the fermentation medium. Each trial was studied by triplicate samples. The yield of bacterial cellulose obtained from all samples was summarized in Table 4.1. The usage of 0 % glucose in the medium was resulted with 0.086 g of bacterial cellulose (dry weight).

The bacterial cellulose yield was increase with the trial of 2 % glucose, which 0.121 g of bacterial cellulose was obtained. However, the yield of bacterial cellulose was decrease and constant at 4, 6, 8 and 10 % of glucose concentration in the medium, where the yield of 0.034, 0.033 and 0.032 g of bacterial cellulose was recorded.

Table 4.1: Weight of Bacterial Cellulose for Glucose Concentration

Glucose (%)	Wet weight (g)	Dry weight (g)
0	19.58	0.086
2	23.021	0.121
4	6.117	0.034
6	4.256	0.033
8	3.773	0.032
10	3.707	0.032

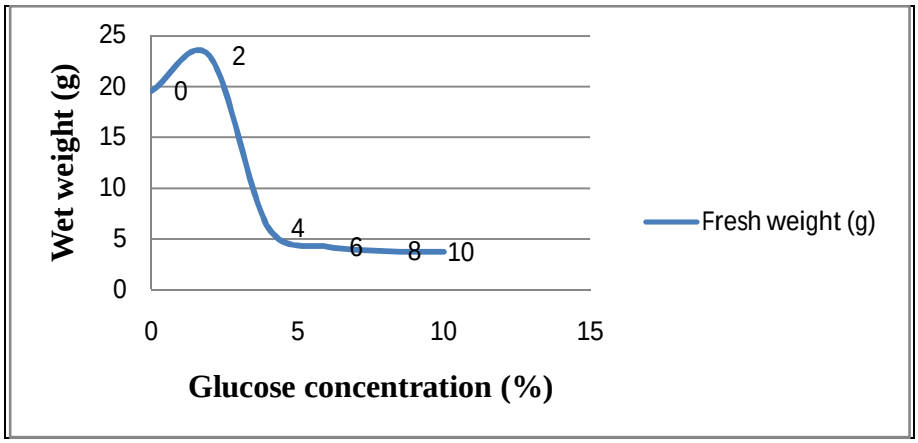

Figure 4.1: Wet Weight of Bacterial Cellulose vs. Glucose Concentration (%)

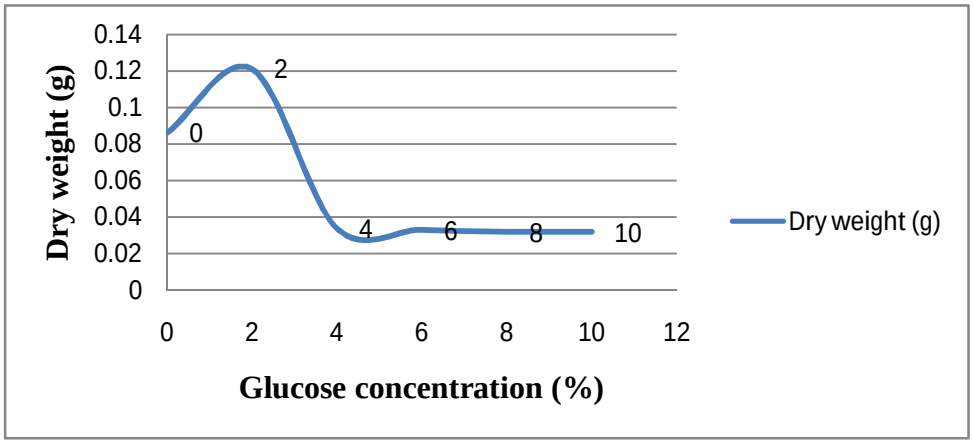

Figure 4.2: Dry Weight of Bacterial Cellulose versus Glucose Concentration (%)

Hence, it can be concluded that the initial concentration of glucose in the medium does reflect on the rate of cellulose production during fermentation process. The rate of cellulose production was increased rapidly from 0 to 2% of initial glucose concentration and decrease at 4, 6, 8 and 10% of glucose concentration. Thus, the minimum range of optimal value for glucose concentration obtained from this study is at 1 to 3%. Similar results were obtained by works conducted previously. Optimum yield of bacterial cellulose at 1 to 1.5% of glucose concentration was reported by Keshk and Sameshima (2005).

Furthermore, most of researchers tend to use about 2% of glucose concentration in preparing medium for bacterial cellulose fermentation in their studies (Clasen et al., 2006). The formation of by-products in the medium during the fermentation process has claimed as the main reason for the reduction of cellulose yield. The consumption of excess glucose concentration during the fermentation process has lead to the formation of gluconic acid in the medium.

When large amounts of gluconic acid present in the medium, the medium were turned into more acidic and yet decreased the pH of the medium. The decrease of medium's pH has affected the growth of *Acetobacter Xylinum* strains and reflected on the rate of cellulose production (Chawla et al., 2009).

4.1.2 Minimum Range of Optimum Value for pH

The minimum range of optimum pH value on the bacterial cellulose fermentation process was studied at pH 4, 5, 6, 7 and 8. The initial pH of each sample was manipulated by adding sufficient amount of acetic acid and sodium hydroxide solution (1M) into the samples. Acetic acid is used to decrease the pH value, while sodium hydroxide is used to increase the pH. The pH of each sample was determined by using pH meter.

The results for each samples is shown in Table 4.2. Based on the results, the highest yield of bacterial cellulose is produced at pH 6 and the lowest yield of bacterial cellulose is produced at pH 8. The dry and wet weight of bacterial cellulose produced at each pH is illustrated in Figure 4.3 and Figure 4.4. The yield of bacterial cellulose is increased from pH 4 until 6. Then, the yield of bacterial cellulose was decreased at pH 7 and 8.

The dry weight of bacterial cellulose produced at pH 6 is 0.322 g and the dry weight of bacterial cellulose produced at pH 8 is 0.174 g. Based on the result, the minimum range of optimum pH value is at pH 5 to 7. Meanwhile, the overall moisture contents of bacterial cellulose produced in the experiments is about 99 %.

Table 4.2: Weight of Bacterial Cellulose at Each pH

pH	Wet weight (g)	Dry weight (g)
4	18.90	0.138
5	19.50	0.227
6	21.59	0.322
7	18.37	0.239
8	16.15	0.174

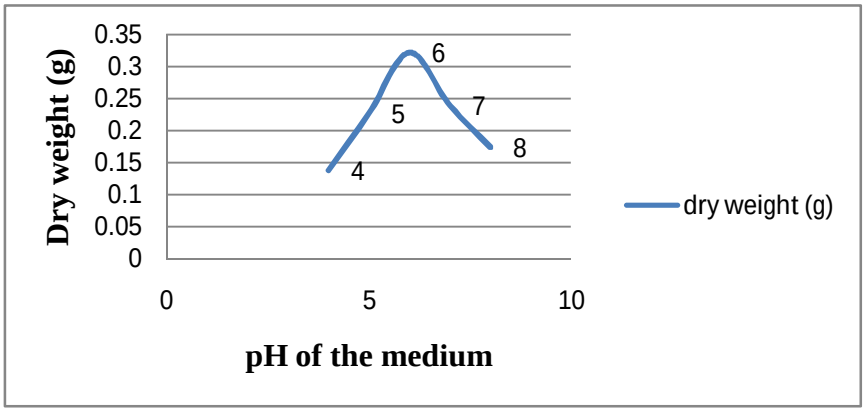

Figure 4.3: Dry Weight of Bacterial Cellulose versus pH Medium

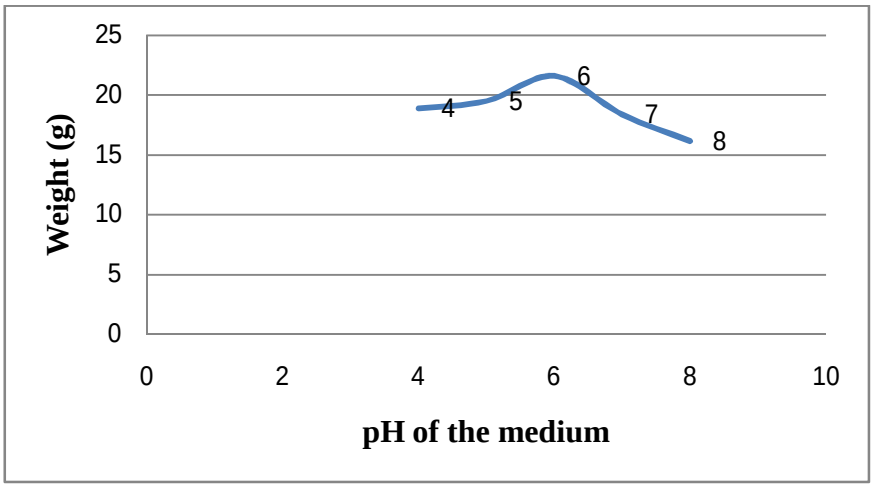

Figure 4.4: Wet Weight of Bacterial Cellulose versus pH Medium

The synthesis of bacterial cellulose during the cultivation period is reflected based on the carbon sources used in the medium. The carbon sources inserted in the medium affected the pH profiles of the medium during the cultivation process. Different type of carbon sources has different effect on the pH profiles during the synthesis process occur during the cultivation (Jung et al., 1999; Keshk and Sameshima, 2005 and Coban and Biyik, 2011).

The study conducted by Verschuren et al. (2000) has revealed that pH at 4 to 5 is the optimum pH range for the cultivation of bacterial cellulose when using sucrose as

the carbon sources. The similar result was obtained by Jagannath et al. (2008) where the optimal pH for fermentation of bacterial cellulose by using sucrose was reported at pH 4.

From the study on final pH of medium which containing glucose, fructose, inositol and glycerol as the carbon sources, Keshk and Sameshima (2005) had showed the relationship existed between yield of bacterial cellulose and type of carbon sources. Compared to the other carbon sources, the medium containing glucose was resulted in sharp pH drop after the fermentation process. Nevertheless, the highest yield of bacterial cellulose was produced from the medium containing glucose as the carbon sources.

The pH changes during the fermentation process were claimed due to the accumulation of gluconic acid as the glucose was consumed rapidly in the cultivation process. The rate of glucose consumption during the cultivation process was reported as 97%. Meanwhile the rate of fructose consumption was only at 52% and 45% for glycerol. The accumulation rate of gluconic acid in the medium was found higher at pH 4 and the rate was significantly reduced with increasing the pH (Jung et al., 1999).

Since *Acetobacter Xylinum* was only capable to synthesis cellulose at minimum pH of 3.5, the usage of higher initial pH is preferred in the cultivation process. The usage of higher initial pH in the cultivation can prevent the surplus accumulation of gluconic acid at the early stages of cultivation process (Coban and Biyik, 2011). Therefore, higher pH such as 5 to 7 is selected as the ideal pH for fermentation of bacterial cellulose by using glucose as the carbon sources.

4.1.3 Minimum Range of Optimum Value for Temperature (°C)

The minimum range of optimum value for the temperature of bacterial cellulose in the study was studied at 25, 27, 29, 31 and 33 °C. The temperature of each sample is manipulated inside the incubator. Table 4.3 showed the wet and dry weight (g) of each sample at various temperatures. The highest yield of bacterial cellulose was observed at

29 °C, where 11.56 g (wet weight) and 0.333 g (dry weight) of bacterial cellulose was recorded.

Table 4.3: Weight of Bacterial Cellulose Produced at Various Temperatures

Temperature (°C)	Wet weight (g)	Dry weight (g)
25	7.827	0.204
27	10.41	0.241
29	11.56	0.333
31	7.507	0.204
33	2.262	0.065

Figure 4.5 and 4.6 illustrated the behaviour of bacterial cellulose production at various temperatures in the study. In the figures, it clearly show that the yield of bacterial cellulose increased linearly from 25 °C until 29 °C. When the temperatures exceed 29 °C, the yield of bacterial cellulose was decreased. Instead of the yield, the quality of bacterial cellulose produced at each temperature also varied. The membrane of bacterial cellulose which produced at 25, 27 and 33 °C was not produced well as the thickness of the membranes was not uniform. The structure of the membranes is not thick as the membranes produced at 28 and 30 °C.

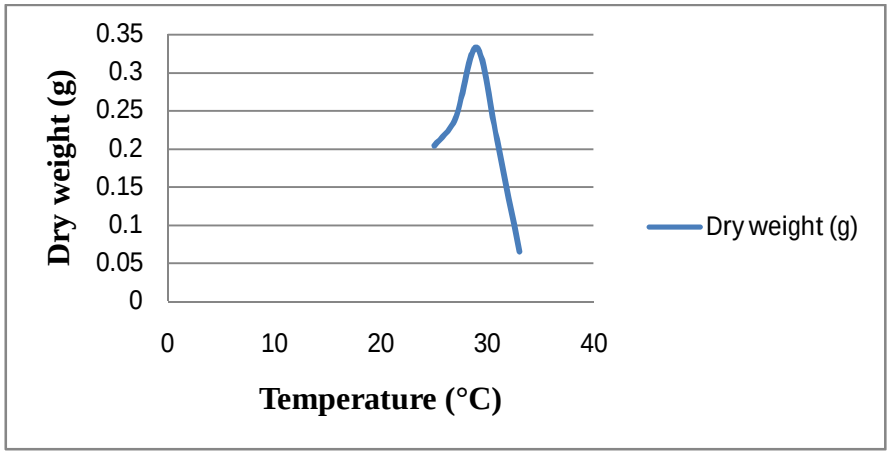

Figure 4.5: Dry weight (g) of Bacterial Cellulose at Various Temperatures

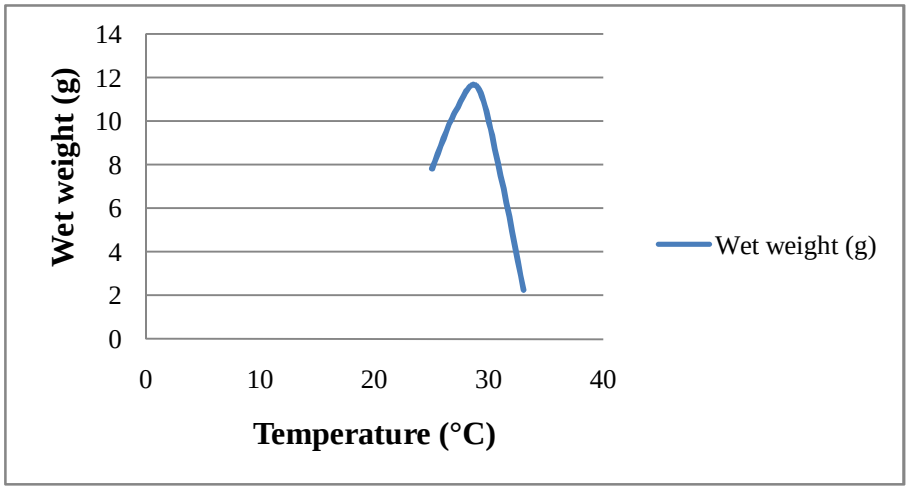

Figure 4.6: Wet Weight of Bacterial Cellulose at Various Temperatures

Basically, the optimum temperature of bacterial cellulose production was believed to be in the range of 28 to 30 °C. The capability of *Acetobacter Xylinum* in producing bacterial cellulose was limited by temperature above 45 °C and the minimum temperature required for the production is 24 °C (Pourramezan et al., 2009).

Despite of the yield, the variation of cultivation temperatures resulted in different degree of polymerisation and water holding capacity (Coban and Biyik, 2011). This is believed to be the reason of instability membrane thickness produced in the present study. The instability and not uniform thickness of bacterial cellulose was observed at 25, 27 and 33 °C in the present study. Therefore, the minimum range of optimum value for the temperature is selected from 28 to 30 °C in the study.

4.2 RESPOND SURFACE METHODOLOGY (RSM)

4.2.1 Central Composite Design (CCD)

The wide application of Central Composite Design (CCD) as the tools to optimize various fermentation processes has become one of the reasons in selecting this program for optimization of bacterial cellulose production in the present study. The optimization processes begin with the design of experimental model, which made based

on the minimum range of optimum value obtained from the previous OFAT experiments.

After completing the experimental model, further statistical analysis by Analysis of Variances (ANOVA) is conducted by the program to examine which variable are significant. Finally, a set of optimized condition for the fermentation process and the prediction on the yield of bacterial cellulose is suggested by the program. The capability of the program in investigating the behaviour of bacterial cellulose production process is determined at the end of the optimization process.

4.2.1.1 Experimental Model

The minimum range of optimum value for variables obtained from previous experiment was inserted into CCD for further analysis. The low and high value for each variable selected based on OFAT results are showed in Table 4.4.

Table 4.4: Ranges of Variable Selected

Variables	Symbol	Unit	Low Level	High Level
Glucose concentration	A	%	1	3
pH	B	-	5	7
Temperature	C	°C	28	30

The three variables of the study are labelled as A for glucose concentration, B for pH and C for the temperature. The predicted optimum value must locate at middle of low level and high level. The selection of optimum value in a wide range is avoided because it maximizes the error in the experimental design (Otto and Wood, 2001).

Based on OFAT results, value of 2 % glucose concentration, pH at 6 and temperature at 29°C was selected as the reference point of the experimental design in CCD. Hence the low and high level of the variables was selected nearest to the reference point, as listed in Table 4.4.

Table 4.5 listed the experimental model designed by CCD based on the optimum value range inserted previously. The experimental design consists of 20 run and each run varied in the variables level. The reference point which is conditions at pH 6, temperature at 29℃ and glucose concentration of 2%, was replicated six times in the experimental model.

Table 4.5: Experimental Design by CCD

Run	Factor A: Glucose	Factor B: pH	Factor C: Temperature	Yields (g)
1	2.00	6.00	30.68	7.10
2	2.00	6.00	29.00	17.30
3	2.00	6.00	29.00	16.10
4	3.00	7.00	28.00	1.47
5	1.00	5.00	30.00	11.40
6	0.32	6.00	29.00	7.60
7	3.00	7.00	30.00	2.10
8	1.00	7.00	30.00	2.75
9	2.00	6.00	29.00	16.40
10	2.00	6.00	29.00	16.50
11	2.00	4.32	29.00	3.50
12	3.00	5.00	28.00	0.20
13	2.00	6.00	29.00	16.90
14	2.00	6.00	29.00	16.50
15	2.00	7.68	29.00	0.30
16	1.00	7.00	28.00	0.29
17	1.00	5.00	28.00	3.85
18	3.00	5.00	30.00	1.26
19	2.00	6.00	27.32	1.50
20	3.68	6.00	29.00	3.80

4.2.1.2 Statistical Analysis

The experimental data obtained from the experiments were used to create mathematical model either by using linear, quadratic and cubic function through application of least squares regression method after which the fitted function are tested for adequacy using ANOVA method.

After the appropriate model is derived, it subsequently used to analyse the optimum condition for the fermentation process. The regression equation used in the mathematical model was then used to generate the response surface plot, which visualized the behaviour of the variables toward the fermentation process in the study (Otto and Wood, 2001).

The statistical analysis of the experimental model started with the selection of the mathematical model based on the interpretation of the experiments data. The model selection was done based on the model analysis, lack fit test and model summary statistics provided by the program. Table 4.6 showed the model analysis, Table 4.7 listed the information of the fit test meanwhile Table 4.8 listed the model summary statistics.

Table 4.6: Model analysis

Source	Sum of Squares	DF	Mean Square	F Value	PROB > F
Mean	1077.81	1	1077.81	-	-
Linear	78.48	3	26.16	0.52	0.6721
2FI	38.09	3	12.70	0.22	0.8829
Quadratic	755.78	3	251.93	480.74	< 0.0001
Cubic	4.09	4	1.02	5.36	0.0350
Residual	1.15	6	0.19	-	-
Total	1955.40	20	97.77	-	-

Table 4.7: Lack of Fit Test

Source	Sum of Square	DF	Mean Square	F value	PROB > F
Linear	798.22	11	72.57	408.44	< 0.0001
2FI	760.13	8.0	95.02	534.80	< 0.0001
Quadratic	4.35	5.0	0.87	4.90	0.0530
Cubic	0.26	1.0	0.26	1.45	0.2820
Pure Error	0.89	5.0	0.18		

Table 4.8: Model Summary Statistics

Source	Standard Deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS Value
Linear	7.07	0.0894	-0.0813	-0.2493	1096.40
2FI	7.65	0.1328	-0.2674	-1.2701	1992.25
Quadratic	0.72	0.9940	0.9887	0.9560	38.60
Cubic	0.44	0.9987	0.9959	0.9337	58.19

From the tables, the quadratic function was selected by the program to represent the response surface of the variables toward the bacterial cellulose yields in the study. The statistic analysis compared the validities of each mathematical function such as linear, cubic and quadratic for different responses based on their F values, PRESS value and probability (Prob>F) value.

The high F value of the quadratic model in Table 4.6 showed the efficiency of the quadratic function in analyse the multiple population variances existed in the experiments data. One of the objectives conducting Design of Experiment (DOE) is to determine whether the variations across the changes in one variable are different from the random error variations. If the different are exist, then the variables are consider as significant (Otto and Wood, 2001).

High value of F test showed the high level of confident that the variation of the changes in particular variable is not because of the randomness. Among the mathematical functions, quadratic showed the highest value of F test. Hence, quadratic are selected to be applied in analyse the significant level of variables in the study.

Inversely, the lowest of F value showed in Table 4.7 indicated that the quadratic model resulted with the minimum error due to the lowest lack of fit value. The lack of fit test showed whether the lack of fit exist between the actual and predicted value can be explain by the experimental error. The model with not significant of lack of fit test is the best to demonstrate the response surface (Ferruh, 2009). Table 4.7 clearly showed that quadratic model has the insignificant value of lack of fit, with Prob>F value exceeds 0.05. Thus, this validates the choice of quadratic as the best function to be applied in the response surface study.

Another indicator for the model selection is the PRESS value which showed in Table 4.8. The PRESS value indicated the efficiency of the particular model match each point in the design. The small value of PRESS value gives a good indication (Otto and Wood, 2001). Since quadratic model resulted with the lowest PRESS value, this mathematical function was selected among the others.

Since the program has determined the best mathematical model to analyse the experiments data, the study was proceed with analysis of variance (ANOVA). The ANOVA of the experiments data was stimulated by using the quadratic model. The significant of the variables and correlation coefficient of the response surface study are determined through ANOVA analysis.

The ANOVA result of the present study was listed in Table 4.9. The variables of the experimental model were justified as the significant variable due to the probability value (Prob>F) less than 0.05 in the analysis. Glucose concentration (A), pH (B) and temperature (C) was significantly affected the bacterial cellulose fermentation process with probability value of 0.001, 0.002 and 0.001.

The interaction of these variables in the fermentation process was also significantly affected the yield of bacterial cellulose produced in the study. Interaction of AB, AC and BC resulted in probability values of 0.001, 0.0023 and 0.0025 respectively.

Table 4.9: ANOVA Results of the Study.

Source	Sum of Square	DF	Mean Square	F Value	PROB > F
Model	872.35	9	96.93	184.96	< 0.0001
A	28.28	1	28.28	53.96	< 0.0001
B	17.55	1	17.55	33.49	0.0002
C	32.66	1	32.66	62.31	< 0.0001
A^2	221.41	1	221.41	422.50	< 0.0001
B^2	399.20	1	399.20	761.77	< 0.0001
C^2	280.86	1	280.86	535.94	< 0.0001
AB	25.63	1	25.83	48.91	< 0.0001
AC	8.65	1	8.65	16.51	0.0023
BC	3.81	1	3.81	7.27	0.0225
Lack of Fit	4.35	5	0.87	4.90	0.050

Besides numerical approach, the significant level of these variables was illustrated in 3D response surface plot and contour plot. These plots were generated based on quadratic regression equation through interpretation of the experiments data. The equation which fitted the experimental model was determined as equation (4.1).

The coefficient value of the equation is determined based on the mean of 95% Confident Interval (CI) low value and 95% CI high value. Table 4.10 showed the estimate coefficient value, the CI value and standard error of the variables.

$$Y = 16.66 - 1.44(A) - 1.13(B) + 1.55(C) - 3.92(A^2) - 5.26(B^2) - 4.41(C^2) + 1.79(AB) - 1.04(AC) - 0.69(BC) \quad (4.1)$$

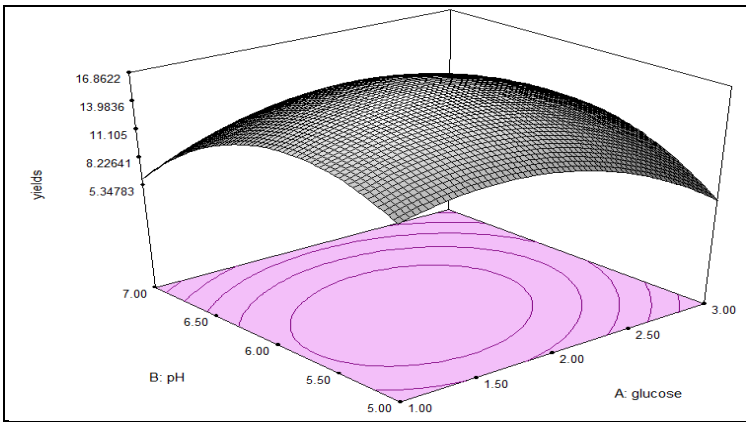

Figure 4.7: Interaction Plot between Glucose and pH

Table 4.10: Determination of Coefficient Value Based on ANOVA Result.

Factor	Coefficient Estimated	DF	Standard Error	95% CI Low	95% CI High
Intercept	16.63	1	0.30	15.97	17.28
A	-1.44	1	0.20	-1.88	-1.00
B	-1.13	1	0.20	-1.57	-0.70
C	1.55	1	0.20	1.11	1.98
A^2	-3.92	1	0.19	-4.34	-3.49
B^2	-5.26	1	0.19	-5.69	-4.84
C^2	-4.41	1	0.19	-4.84	-3.99
AB	1.79	1	0.26	1.22	2.36
AC	-1.04	1	0.26	-1.61	-0.47
BC	-0.69	1	0.26	-1.26	-0.12

The response surface and contour plots represent the relation between the bacterial cellulose yield and the variables in the study. The curvature shape of the response surface around the reference point was due to the elliptical contour plot system. The elliptical contour plot reflected from the maximum response around the reference point (Ferruh, 2009). In other word, the prediction of reference point at glucose 2%, pH at 6 and temperature 29°C are almost reach the maximum response of bacterial cellulose yield in the fermentation process.

Theoretically, the curvature shape of the response surface in Figure 4.7 is believed to be reflected by the significant effect of the variables AB toward the bacterial cellulose production process since the coefficients of the response function (regression equation) were determined by least square method using data from the chosen data set of variables and measured their responses. Since the coefficients of the regression equation determined from the experiments data was resulted with maximum response surface, the interaction of AB are believed to enhance the optimization of the bacterial cellulose yields significantly.

Graphically, the curvature along the glucose and pH axis in Figure 4.7 showed that the variations of these variables were resulted in maximum and minimum yields of the bacterial cellulose in the fermentation process. The significant maximum and minimum yields of bacterial cellulose yields reflected from the variables changes were statistically measured with significant probability value of 0.001.

The significant effect on bacterial cellulose yields which introduced by the interaction of glucose concentration and pH was due to the connection existed between these two variables. Any single changes made on the glucose concentrations of the medium do react on the pH of the medium during the fermentation process. Since *Acetobacter Xylinum* was sensitive with the pH changes during the synthesis process, the yields of bacterial cellulose produced from the study was greatly affected with changes of AB introduced in the fermentation process.

Acetobacter Xylinum was capable to produce bacterial cellulose in initial pH range from 4 to 6, where there is no formation of bacterial cellulose occur in initial pH below 3.5 and above pH 7 (Chawla et al., 2009; Zippora and Hestrin, 1962). The pH changes during the fermentation were related with the type of carbon sources used in the medium (Kesk and Sameshima, 2005).

The usage of glucose as the carbon sources in the fermentation medium resulted with significant pH drop due to the rapid glucose consumption during the fermentation process. The accumulation of gluconic acid from the glucose consumption by the strain was affected the pH of the medium (Thompson and Hamilton, 2000). Hence, the suitable amount of glucose and initial pH of the medium has to be select accurately to avoid excess pH drop and at the same time to supply sufficient amount of carbon sources in order to achieve the optimum yields of bacterial cellulose from the fermentation process.

The interaction of glucose with temperature was also resulted with significant effect toward the bacterial cellulose yields in the fermentation process. The response surface and contour plot of their interaction are illustrated in Figure 4.8. Interruptions in the temperature are mainly affected the *Acetobacter Xylinum* kinetic growth during the fermentation process.

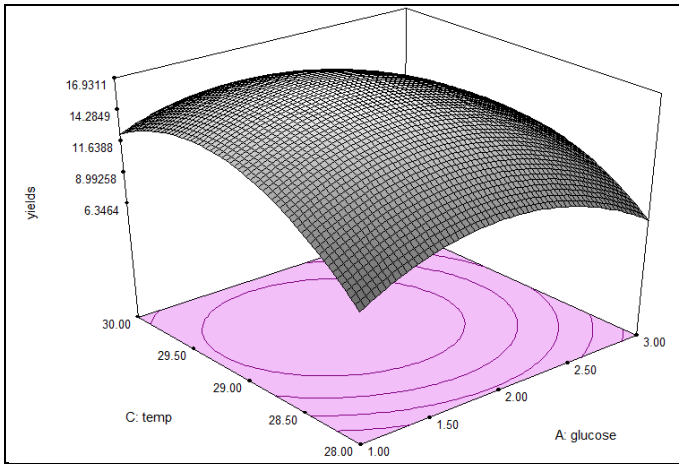

Figure 4.8: Interaction Plot between Glucose and Temperature

The other effect resulted from the interruption is the changes on behaviour of the bacteria and the oxygen dissolution in the fermentation medium. The acetification of the bacteria in the fermentation was also disrupted by the interruption made on the incubation temperature (Vaughn, 1938).

Acetobacter Xylinum was reported capable to grow within temperature 25°C to 30°C during the fermentation process. Nevertheless, the comparison of bacterial cellulose produced at 28°C to 30°C has resulted into quadratic curve in the Figure 4.8. The bacterial cellulose yields increase along the curve from 25°C and upward due to the synthesis process occur rapidly with the faster rate driven by the temperature rises and linear kinetic growth. After reach above the reference point at 29°C, the yield was irreversibly decreased due to disruption at higher temperature.

The variation of temperature does affect the bacteria growth, which then resulted into less glucose consumption during the fermentation process. Without sufficient utilization of carbon sources during the fermentation process, the synthesis of bacterial cellulose was disrupted and not efficient at particular condition (Keshk and Sameshima, 2005).

The yield of bacterial cellulose decreased from the temperature disruption was also believed due to the reduction of water binding capacity and degree of

polymerisation of the bacterial cellulose. Bacterial cellulose produced at 30°C was resulted with better water binding capacity as compared to production of bacterial cellulose at 25 and 35°C (Coban and Biyik, 2011). Hence, the variation in temperature and glucose concentration was proved significantly affected the yields of bacterial cellulose in the study.

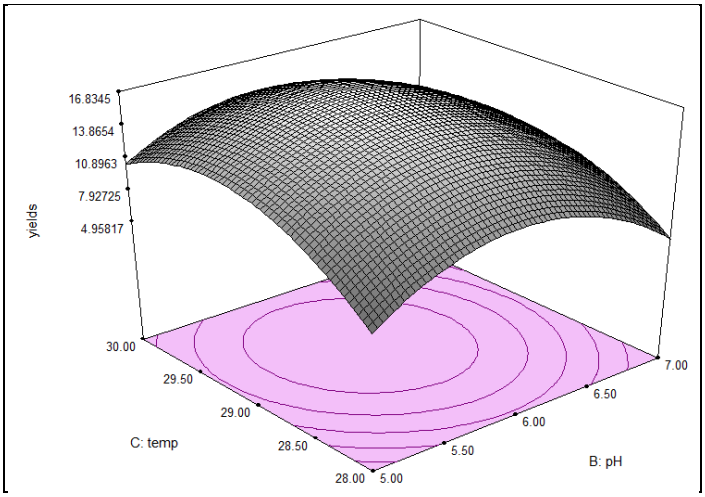

Figure 4.9: Interaction Plot between Temperature and pH.

Similar significant pattern was observed in Figure 4.9, where the interaction of bacterial cellulose was also significantly affected the yields of bacterial cellulose during the fermentation process. The probability value of their variation in the experiments data was 0.0025, which less than 0.05.

As mentioned before, the variation of temperature disrupted the kinetic growth of the bacteria meanwhile the variation in pH resulted with acidity of the medium during the fermentation process. Although there is no connection observed between these two variables, but the variation on these two variables still resulted into significant effect on the bacterial cellulose yields during the fermentation process.

The yields of bacterial cellulose significantly increase from pH 5 to pH around 6, after which it was decrease after pass through the reference point at pH 6. This observation was consistent to the result of combination pH with glucose concentration and combination between temperature and glucose concentration previously. Hence, pH

and temperature are proved significantly affected the yield of bacterial cellulose from fermentation process.

The R^2 of the overall experimental model was resulted with value of 0.994. The adjusted and predicted R^2 of the experimental model resulted with value of 0.9887 and 0.9560. The deviation of the adjusted and predicted R^2 is around 0.03. This satisfied the requirement of deviation value below 0.20 respectively (Design Expert, 2000).

The R^2 value gives indication on the overall linearity of the experimental data with the predicted yield from CCD. The R^2 value is ranged from -1 to +1. Zero value of R^2 indicates that no correlation exist in the data, meanwhile ±1 value of R^2 shows whether it's a positive or negative linearity. The R^2 approximate to 1 indicates that the best linear fit (Nermeen et al., 2010).

4.2.1.3 Diagnostics of the Experimental Model

The efficiency of the designed experiment model was observed on the Box-Cox plot, as showed in Figure 4.10. Based on the plot, current experiment model is approximately near to achieve the best model as the Lambda value of the current model is at 1. Meanwhile the Lambda value for the best experiment model is at 0.74. There is no recommended transform from the analysis. Thus, the current experiment model is suit well to the optimization process.

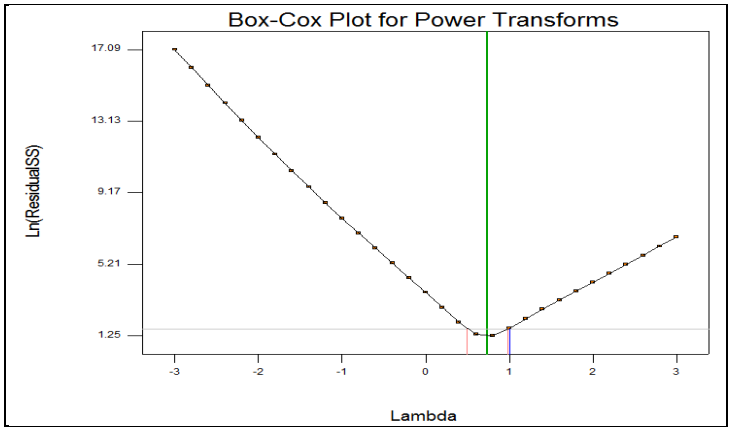

Figure 4.10: Box-Cox Plot of the study

The key factor to investigate the effectiveness of the experiment model in identify the behaviour of the bacterial cellulose production process is based on the plot of predicted value versus actual value. The significant effect of the variables is believed to be related with the compatible degree of the predicted and actual value of bacterial cellulose's yield which resulted from the experimental model.

Figure 4.11 clarifies the plot of predicted versus actual value of bacterial cellulose yield in the present study. The linear plot of the predicted and actual yield of bacterial cellulose in the figure shows the high precision of quadratic model in fitting the experimental model.

The actual value was proportional with the predicted value of the program because there are less error existed between the predicted and actual yield of bacterial cellulose produced from the experiment design. Hence, the program are able in optimizing the production of bacterial cellulose in the study due to its capability in identify the behaviour of the bacterial cellulose production process with less error.

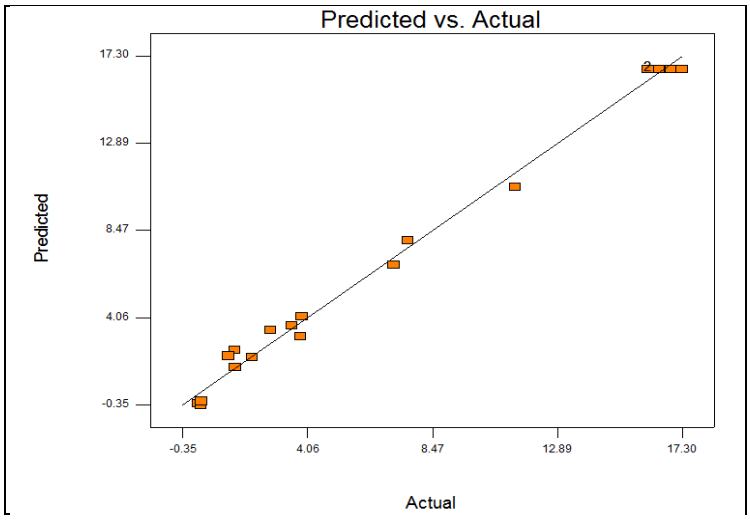

Figure 4.11: Plot of Predicted vs. Actual Value

The residual of the predicted and actual value of the study were further analysed with normal probability percent. Figure 4.12 illustrated the plot of normal probability percent versus the residuals. The approximate linear plot of these values in the figure

indicated that there adequate normality in term of error present between the predicted and actual value. The non-normality of error existed in the residuals are claimed to be resulted with the curve or S-shape plot (Design Expert, 2000).

Since the present plot are approximately linear, the error normality exist in the study are acceptable and not too bad. Thus, the experimental model designed by the program are fit due to its capability in identify the behaviour of bacterial cellulose production process with the adequate normality in the term of error present from the experiment.

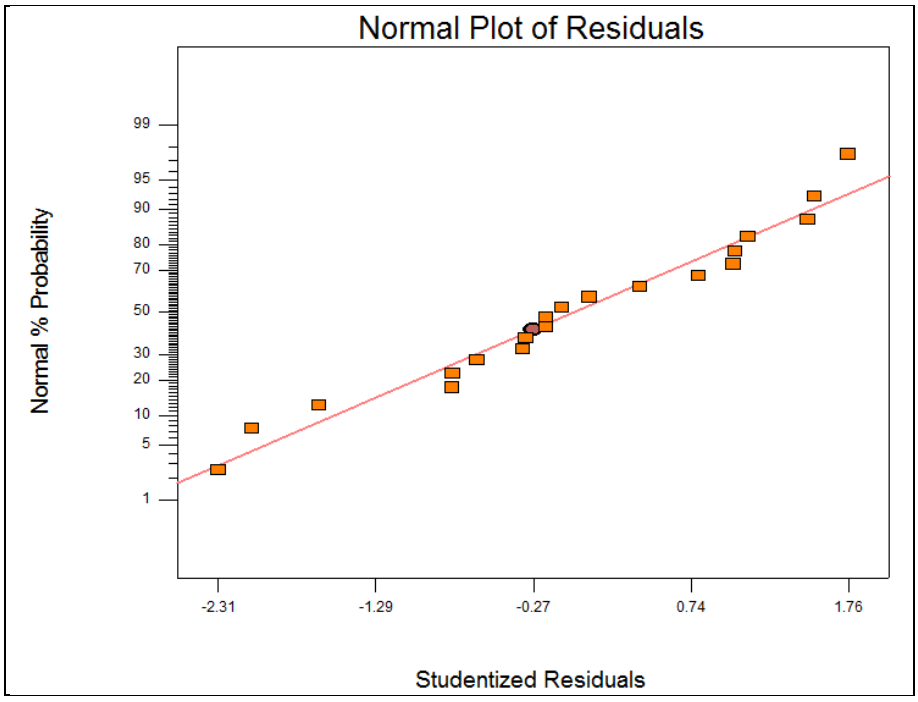

Figure 4.12: Plot of Normality % Probability vs. Residuals

The overall standard error of yields resulted from the experiment model are illustrated in Figure 4.13. In the figure, the error of yields is narrowing down as the variables approaches to the reference point. The reference point is the optimum value of variables chosen from OFAT experiment. The reference point for pH is 6, 2% for glucose concentration and 29°C for temperature.

The standard error was increase after the variables passed through the reference point, as illustrated in the figure. The lowest value of standard error for bacterial cellulose yields at the reference point indicated that the selection of pH at 6, glucose concentration at 2% and temperature at 29°C as the reference point in the study are precise and almost reach to the optimum yield of bacterial cellulose from the fermentation process. The standard error value of the reference point was 0.4006 and the overall standard error of the experiment model was range from 0.4006 to 0.6023.

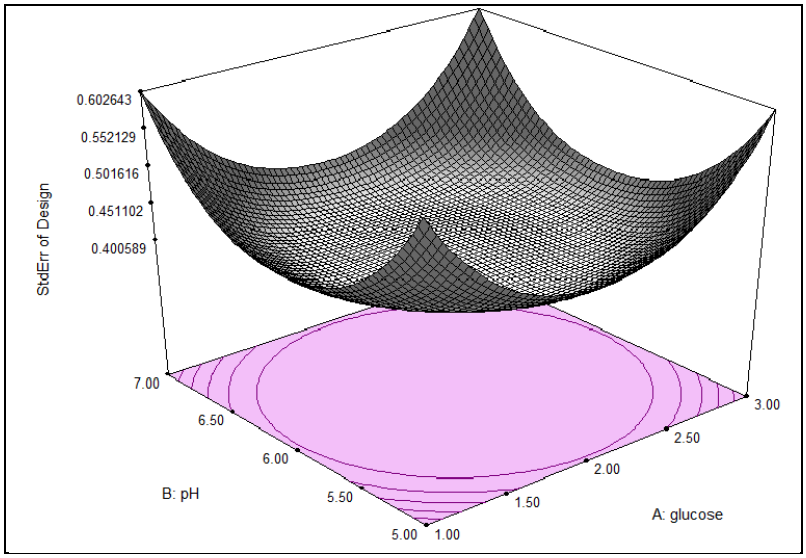

Figure 4.13: Standard Error Plot of Overall Experimental Model

4.2.1.4 Optimization Process

The study was continued with the optimization process on the significant variables by investigates the specific optimum value for each variable. The numerical optimization of the variables was showed in Table 4.11. Based on the minimum range inserted earlier and by conducting multiple cycles to find the optimums, the program finally select a set of optimum conditions to achieve desirable yield of bacterial cellulose.

The top of the table shows the minimum ranges of optimum value for each variable which has been inserted in the design earlier. The bottom of the table shows the multiple cycles that conducted by the program to improve several conditions that

perhaps can be use to achieve desirable results. Out of ten conditions, the program finally comes out with only one set of selected conditions.

The selected conditions suggest the specific optimum value for each variable. The specific optimum value suggested for glucose concentration is 1.75%, pH at 5.84 and temperature at 29.22°C. By following these conditions, the program predicted that 17.07 g of bacterial cellulose will be produced in the fermentation process.

Table 4.11: Optimization of Variables.

Solution Selected					
Number	**Glucose (g/l)**	**pH**	**Temperature (°C)**	**Yields (g)**	**Desirability**
1	1.75	5.84	29.22	17.067	0.986
Number of Starting Points 10					
1	2.66	6.94	29.39	-	-
2	1.01	5.66	28.03	-	-
3	1.43	6.14	29.82	-	-
4	1.68	5.44	29.99	-	-
5	1.33	5.89	29.83	-	-
6	1.08	5.26	29.52	-	-
7	2.68	5.20	28.22	-	-
8	1.72	5.75	29.49	-	-
9	2.49	6.62	29.57	-	-
10	2.53	5.85	28.27	-	-

4.2.1.5 Verification of the Optimization Process

The final fermentation of bacterial cellulose was conducted by following the optimum value suggested by the program. The medium was prepared by using 1.80% of glucose, the initial pH was adjusted to 5.84 by adding Sodium Hydroxide (NaOH) and the sample was incubated at 29.22°C.

After 3 days of fermentation, the bacterial cellulose was treated by NaOH to eliminate the bacteria and the weight of bacterial cellulose was measures. For comparison and to determine the optimization scale of present study, there are another sample of bacterial cellulose by following conventional method was prepared. Table

4.12 listed the yield of bacterial cellulose produced from both present and conventional method.

Based on the result, the present study was capable to produced 17.81 g of bacterial cellulose by following the optimize conditions suggested by the program. Meanwhile, the conventional method was only capable to produce 4.94 g of bacterial cellulose. Therefore, the yield of bacterial cellulose in the present study was optimized 3.602 times higher than conventional method.

Table 4.12: Optimized Yields of Bacterial Cellulose

Sample	**Conventional method**		**Optimized method**	
	Dry weight (g)	**Wet weight (g)**	**Dry weight (g)**	**Wet weight (g)**
i.	0.062	5.05	0.212	19.05
ii.	0.055	4.79	0.165	17.38
iii.	0.040	4.98	0.141	17.01
Mean weight (g)	0.157	4.94	0.562	17.81

The same optimization was observed based on the dry weight of bacterial cellulose. Collectively, 0.562 g of dry weight bacterial cellulose was produced by following optimized method, as compared to 0.157 g of dry weight bacterial cellulose was produced by following conventional method.

The experimental model which designed by the CCD program in the present study was able to identified the behaviour of bacterial cellulose production process as the actual value of bacterial cellulose produced from the optimization is approximate to the predicted value, where the program predicted 17.07 g of bacterial cellulose will be produced by following the optimized condition. The error of the program prediction on yield of bacterial cellulose from the optimization process is 4.33 %. The error was calculated by followed the equation (4.2).

$$\text{Error} = \frac{\text{Actual yield} - \text{Predicted yield}}{\text{Predicted yield}} \qquad (4.2)$$

Thus, the experimental model designed by the program was successfully optimized the yield of bacterial cellulose production with 3.6 times higher than conventional method. Based on the probability value of ANOVA analysis, the three variables examined in the study was significantly affected the bacterial cellulose fermentation process.

The optimum value of glucose concentration was found at 1.75%, initial pH is at 5.83 and temperature was found significantly affected the bacterial cellulose fermentation process at 29.22 °C. The experimental model was significantly optimized the bacterial cellulose production by the final fermentation process with 98.7% desirability. Hence, the application Response Surface Methodology was relevant in optimizing the fermentation process of bacterial cellulose.

A study on optimization of bacterial cellulose process conducted by Jaganath et al. (2008) was resulted with pH as the significant variables. The ammonium and sucrose concentration was not significantly affected the yield of bacterial cellulose. Nevertheless, the optimum condition was identified at pH 4, 10% of sucrose and 0.5% of ammonium sulphate concentration. The author claimed that the optimal pH of bacterial cellulose fermentation process depend on the type of carbon sources supplied to the medium.

Since utilization of glucose was found optimal at pH 5.83 meanwhile the utilization of sucrose in the fermentation process was resulted with pH 4 as the optimal pH, it proved that the optimal pH is reflected by the type of carbon sources used in the fermentation process.

4.3 ANALYSIS OF BACTERIAL CELLULOSE

4.3.1 Fourier Transform Infrared (FTIR) Spectrophotometer

The analysis on molecular fingerprint of the bacterial cellulose produced from the optimization process was carried out by using FTIR spectrophotometer. The

spectrum of absorption peaks resulted from the FTIR analysis represent the type of atom and bond of particular compound that exist in the sample (ThermoNicolet, 2001).

Generally, the main compound that researchers wish to observe in bacterial cellulose sample is the content of Cellulose compound, which located in the crystallin region in the sample (Monika et al., 2011). Instead of cellulose content, the hydrogen bonding and miscibility that exists in the sample also become one of interest when conducting FTIR spectra analysis on modified bacterial cellulose sample (Zhang et al., 2011). The present of several type of functional groups such as hydroxyl groups, C-H bond, C-O ether bond of the sample analysis indicated that the typical spectrum of cellulose (Vazquez et al., 2006).

In the present study, analysis of FTIR was resulted with observation of Cellulose Iß compound, C-H bond, C-H stretch bond, C-O ester bond and hydroxyl bonds. Figure 4.14 illustrated the FTIR spectrum resulted from the analysis. In the figure, the spectrum at 896.62 responding to the presence of crystal structure cellulose Iß compound, meanwhile the spectrum at 1028.10 reflected by C-O ester bond in the sample. The existence of C-H bond and C-H stretch bond is showed by the spectrum at 1370.45 and 2902.61 in the figure. The highest spectrum which is 3331.48 indicated the presence of hydroxyl groups.

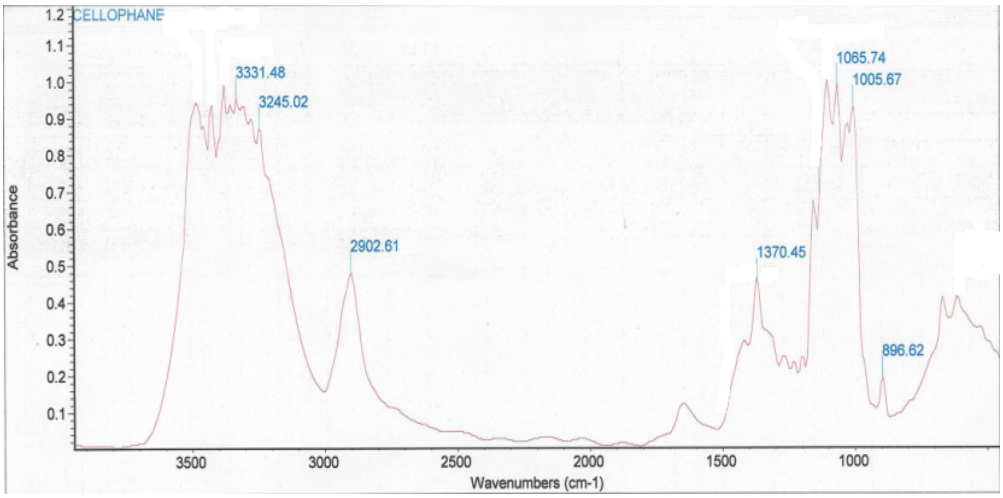

Figure 4.14: FTIR Spectrum of Cellulose in Bacterial Cellulose

The presence of these compounds was justified based on the characteristic IR absorption at particular range of wavenumber. Each compound has specified range of wavenumber based on the functional group absorption in the spectrum. Wavenumber ranged from 3200 to 3500cm^{-1} indicated the presence of hydroxyl groups, 2850 to 3000 cm^{-1} indicated the presence of C-H stretch bond, 1350 to 1370cm^{-1} indicated the presence of C-H bond and wavenumber of 1000 to 1320cm^{-1} reflected by C-O stretch bond in the sample (Spectroscopy Tutorial, 2011).

Previously, the content of Cellulose Iβ compound in bacterial cellulose sample was reported found at wavenumber of 897cm^{-1} (Spiridon et al., 2010). Basically, *Acetobacter Xylinum* strain was claimed to have capability in producing compound of Cellulose II naturally. The same observation was found in the bacterial cellulose produced by the algae. Nevertheless, the transformation of Cellulose Iβ into Cellulose II inside the bacterial cellulose has been investigated by Klemm et al. (2001). The transformation of Cellulose Iβ into Cellulose II was claimed due to the mercerization process, which occurred by treatment of bacterial cellulose with high concentration of sodium hydroxide (NaOH) solution (17-20 % w/v).

In the present study, the bacterial cellulose obtained from the fermentation was treated with 1 % of NaOH as a way to eliminate the existence of bacteria on the surface of the sample. Due to the low concentration of NaOH was used in the treatment, the transformation of Cellulose Iβ into Cellulose II was not happen. This explains the reasons of formation Cellulose Iβ compound inside the bacterial cellulose and its relation with Cellulose II which claimed it was produced naturally by *Acetobacter Xylinum* strain.

According to Gea et al. (2011), the transformation of cellulose I to cellulose II which caused by application of NaOH over concentration 6% during the purification process of the sample instantaneously lead to lower mechanical strength of the bacterial cellulose membrane. The mechanical properties was lowered after the treatment due to the inter and intra molecular hydrogen bond of the bacterial cellulose was damaged during the purification of the membrane with high concentration of NaOH. Hence, the

selection of purification of bacterial cellulose with lower concentration in the present study is relevant to its effect toward the mechanical strength of the bacterial cellulose.

In addition, further treatment of bacterial cellulose with glycerol and ammonia can lead to the transformation of Cellulose I into Cellulose III and Cellulose IV. The transformation of cellulose into various types of polymorphs is illustrated in Figure 4.15.

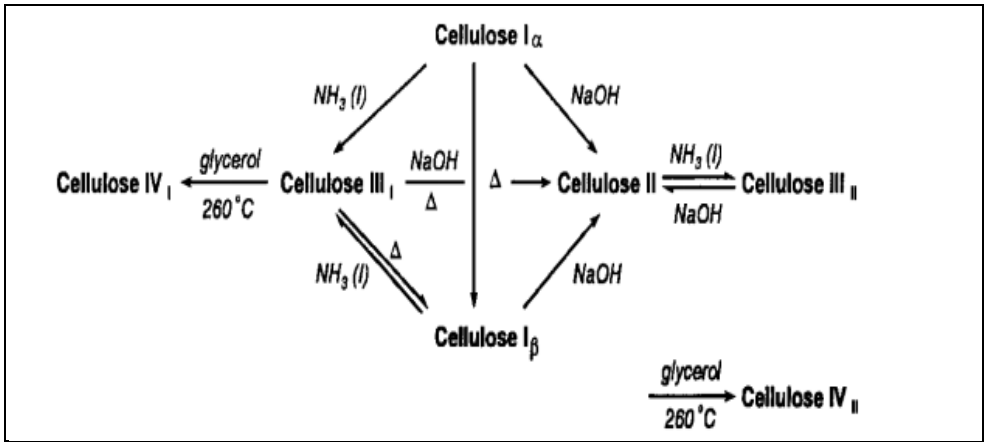

Figure 4.15: Reactions Related to Cellulose Changes

Source: Klemm et al. (2001)

Despite of Cellulose compound, the other main compound observed under FTIR spectra in the present study is D-glucose crystalline compound. Basically, the cellulose molecule was claimed exist as unbranched polymer which linked together with D-glucose units by β-1,4 glycosidic bonds (Monika et al., 2011). The spectrum of D-glucose compound resulted from the FTIR analysis is illustrated in Figure 4.16.

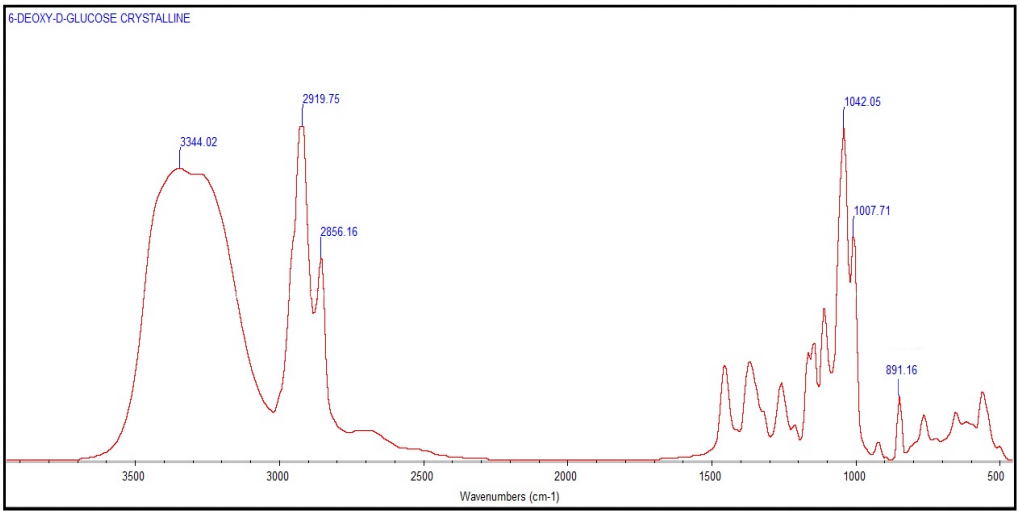

Figure 4.16: FTIR Spectrum of D-glucose in Bacterial Cellulose.

Surprisingly, there are another polymer compound were observed from the FTIR analysis, which identified as Poly (methylphenylsiloxane) or known as PMPS. PMPS is a polymer which classified under silicone polymer and was claimed as a desirable blend component in making nanocomposites material (Brande et al., 2011).

Meanwhile, the stereochemical chain of PMPS was reported identical with such as polystyrene and poly(α-methylstyrene) (Mao and Hillmyer, 2008). The spectrum of PMPS matched with the bacterial cellulose spectrum in the present study is showed in Figure 4.17.

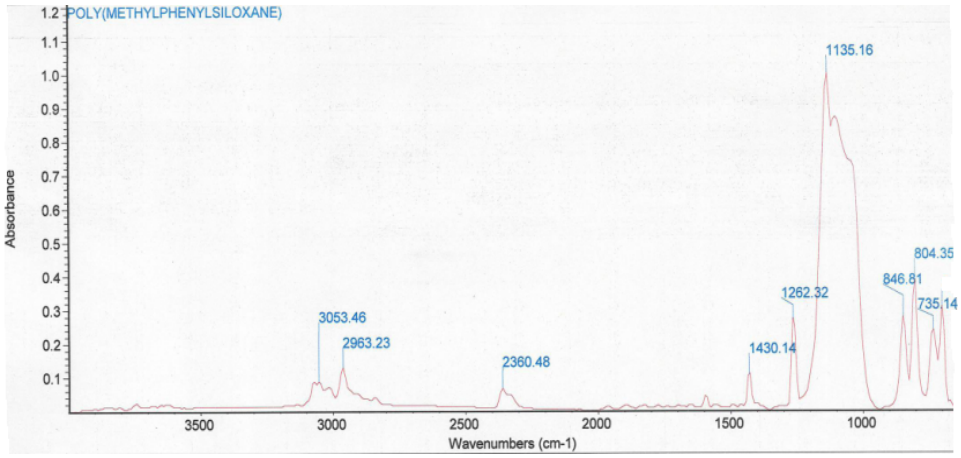

Figure 4.17: FTIR Spectrum of PMPS in Bacterial Cellulose.

The observation of others polymer compound besides of cellulose in the bacterial cellulose sample has been reported by Tajima et al. (1997). In his study, the production of bacterial cellulose by using sucrose as the carbon sources was resulted with production of water soluble polysaccharide (WSP), which identified to be linked by ß-(2→6)-linked polyfructan.

This polymer was claimed having different structure with the polymer synthesized by using glucose as the carbon sources in the cultivation of *Acetobacter Xylinum* strain. Tajima et al. (1997) also claimed that his study has revealed the new findings on capability of *Acetobacter Xylinum* in producing different type of polysaccharides.

The physical properties of compound existed in the bacterial cellulose sample were successfully analysed under FTIR spectra, where the spectrum of cellulose and D-glucose units were determined from the analysis. The cellulose spectrum which contains the main material of cellulose compound such as C-H bonds, hydroxyl bonds, C-O ester bond and crystal structure of cellulose Iß has confirmed that the sample produced in the present study is a cellulose compound.

4.3.2 Scanning Electron Microscope (SEM)

The morphology of the surface and cross section of bacterial cellulose sample produced in the study was investigated by using scanning electron microscope (SEM). The investigation was conducted to illustrate the structure and morphology of the fibre networks which believed contained in the bacterial cellulose.

Figure 4.18 illustrated the cross section morphology of bacterial cellulose taken at 100 x magnifications, meanwhile Figure 4.19 visualized the cross section morphology at 500 x magnification of SEM. The observation on cross sections showed that the sample consists of layered fibre of the cellulose network. The formation of the layered structure was claimed due to overlap of fibres across the thickness of the bacterial cellulose membrane (Jia et al., 2010).

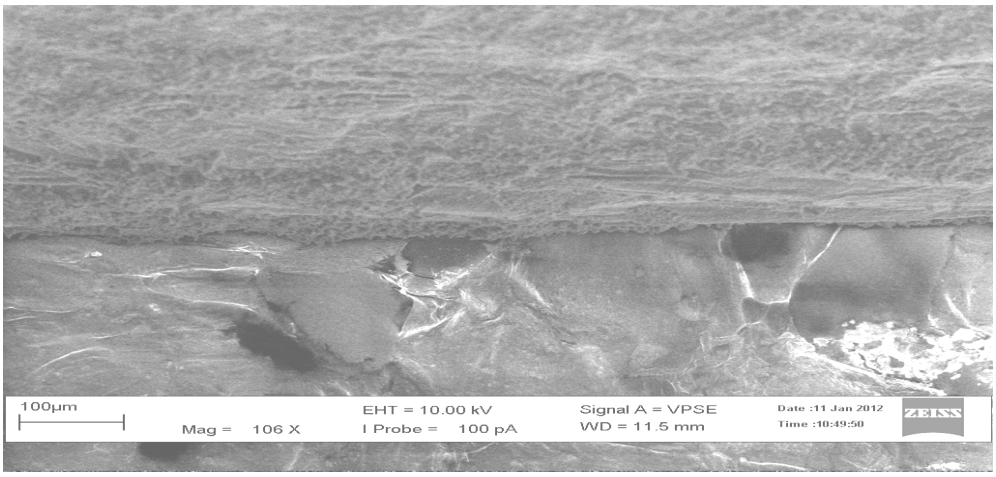

Figure 4.18: Cross Section of Bacterial Cellulose at 100 x magnification.

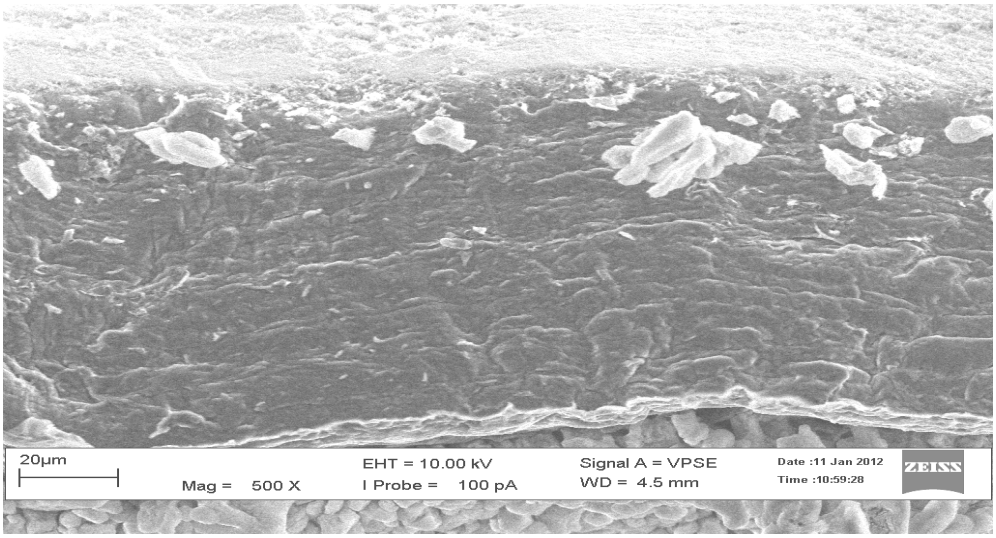

Figure 4.19: Cross Section of Bacterial Cellulose at 500 x Magnification

The nutrients supply and cultivation time was believed as the main factor that contribute to the thickness of bacterial cellulose produced from the fermentation process. This condition resulted from the fact that the production of bacterial cellulose is proportional with the cultivation days, where the increase of cultivation days required more nutrients supply to support the cell growth during the fermentation process (Verschuren et al., 2000).

High cultivation time and high nutrients supply to the medium generates more production rate of bacterial cellulose because the bacteria gained more energy to reproduce the cellulose membrane with sufficient doubling time of the cell growth during the fermentation process (Chawla et al., 2009).

Hence, better observation of the fibre networks across the thickness of the membrane can be achieve when long cultivation period and sufficient nutrients supply during the fermentation as more thickness of cellulose membrane formed during the long fermentation process.

On the other hand, the surface morphology of the sample is showed in Figure 4.20. The fibre network of the cellulose was not observed from the surface morphology of the bacterial cellulose. The absent of the fibre on the bacterial cellulose surface may caused by the effect of cultivation time and nutrients supply to the medium.

In the present study, the cultivation of the fermentation process was carried out within three days only. Moreover, the other nutrients supply such as yeast extract, peptone and magnesium sulphate was not inserted in the medium. Thus, the lack of nutrients and time may affect the quantity of fibre formation in the bacterial cellulose membrane as the bacteria has lack of time to grow well and might be affected by the lack of additional nutrients.

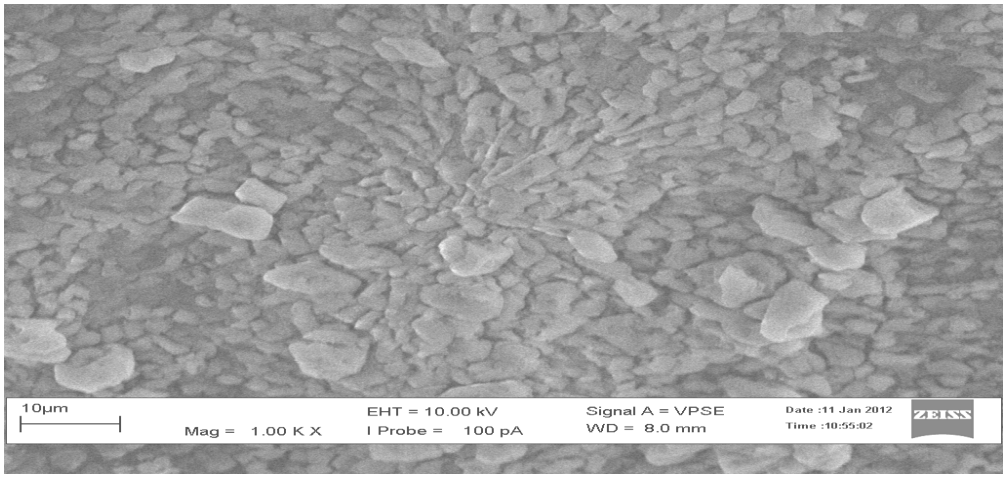

Figure 4.20: Surface Morphology of Bacterial Cellulose.

The high resolution of bacterial cellulose fibre network structure conducted by Gu et al. (2010) was successfully demonstrated the clear morphology of the bacterial cellulose, which was observed under 10,000 x magnification of SEM. The fermentation of bacterial cellulose in the study used coconut water with addition of peptone, yeast and magnesium sulphate. Moreover, the fermentation was conducted within five days. It might be the reason of the well formation of fibre inside the bacterial cellulose.

The formation of the fibre networks inside the bacterial cellulose is reflected on the mechanical strength of the bacterial cellulose, where the Young Modulus of the bacterial cellulose with denser fibre networks was greater as compared with the less one (Gea et al., 2010).

The mechanical strength of the bacterial cellulose membrane was also claimed depend on the quantity of the hydrogen bond formed in the bacterial cellulose. The high quantities of hydrogen bonds accelerate more formation of fibre networks with high porosity inside the membrane of bacterial cellulose (Gu et al., 2010). Thus, the membrane produced with great thickness will exhibit denser fibre networks and perform good quality of mechanical strength since it contained more hydrogen bond across the membrane thickness.

Nevertheless, the purification process of the bacterial cellulose with NaOH after the fermentation process was claimed as the external factor that contributed to better intra-connection between the hydrogen bonds inside the bacterial cellulose morphology, since the purification process helps to remove the impurities resulted from the medium and bacterial cell after the fermentation process. It can leads to more clear observation of the fibre networks on the surface and cross section of the bacterial cellulose (Gea et al., 2010).

In conjunction with that claims, further observation by using SEM on the untreated bacterial cellulose in the present study were conducted. Figure 4.21 visualized the cross section part meanwhile Figure 4.22 visualized the surface morphology of the untreated bacterial cellulose.

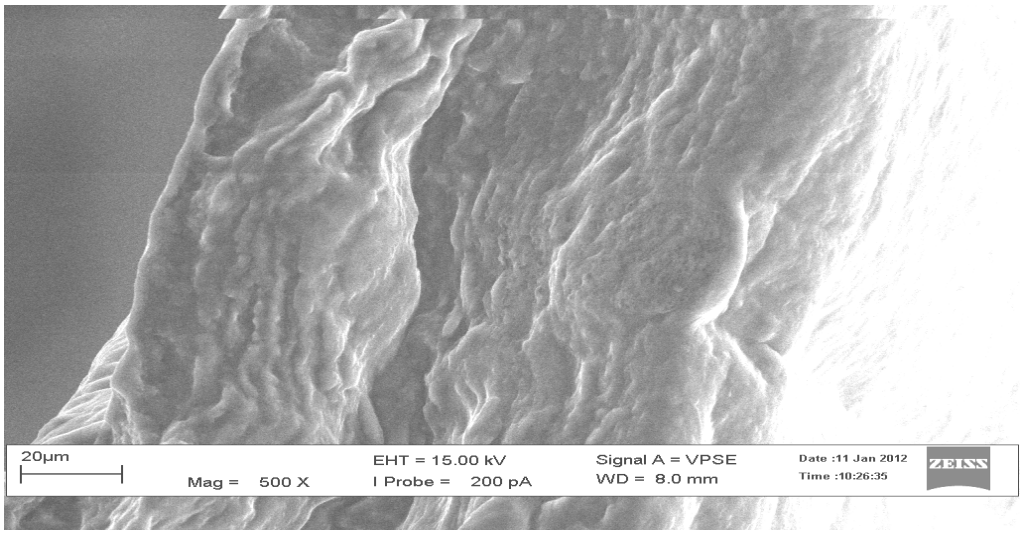

Figure 4.21: Cross Section of Untreated Bacterial Cellulose.

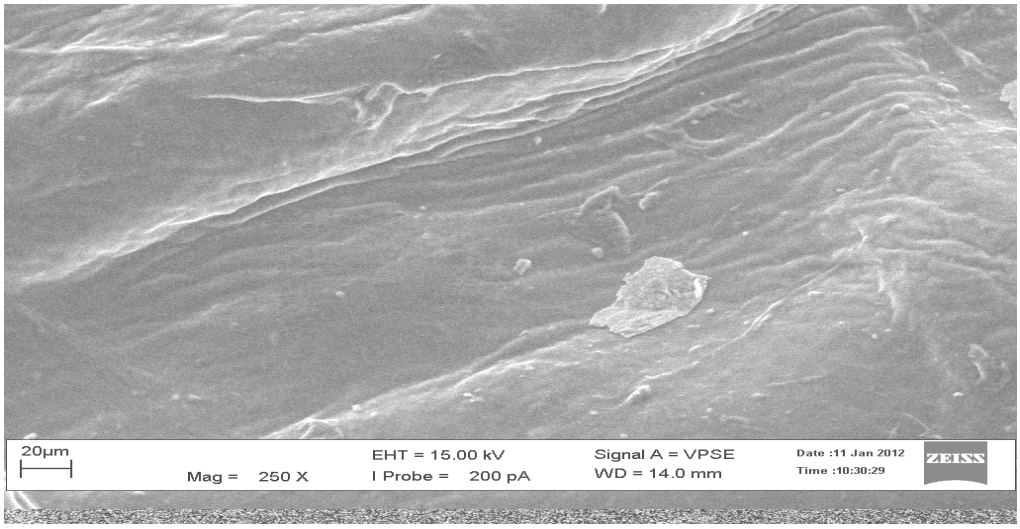

Figure 4.22: Surface Morphology of Untreated Bacterial Cellulose.

From observation on both of the figures, the bacterial cellulose surface looks like coated by a layer and looks smooth. The layer makes the surface and the cross section of the bacterial cellulose looks like flat and without any fibre networks. The outer layer which responsible for the fibre network absent is believed to be the impurities resulted from the fermentation process, such as proteins, vitamins and food sources in the medium (Gea et al., 2011).

In her study, Gea et al. (2011) has demonstrated that the analysis of untreated bacterial cellulose was resulted with opaque images, where the surface of the untreated bacterial cellulose was covered with impurities. The treatment of bacterial cellulose with two steps, which is the treatment with 2.5% of NaOH and 2.5% of NaOCI was proposed in her study since the treatment of high concentration of NaOH potentially leads the transformation of polymorph cellulose I to cellulose II due to the inter and intra hydrogen bond connection was damaged (Klemm et. al., 2001).

The treatment of bacterial cellulose with two step alkalization was resulted with clear observation under SEM analysis and yet the inter and intra hydrogen bond of the bacterial cellulose still protected from being damaged. Clear observation of fibre networks was observed as compared to the treatment with single step purification with 2.5% of NaOH. The two step purification also enhanced the mechanical strength of the bacterial cellulose since the elimination of the impurities allowing better connection between the fibre networks and increases the Young modulus value (Gea et. al., 2011).

Thus, the two treatment of bacterial cellulose with 2.5% of NaOH followed by 2.5% of NaOCI is proposed in order to observe more clear fibre network within the bacterial cellulose.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.0 INTRODUCTION

The last chapter of the thesis is about the summary of all findings from the present study. Previous chapter has elaborated the results and discussion regarding to all findings in the study. Thus, this chapter summarized all of the findings based on two main scopes, which are the conclusion and further recommendation based on the findings.

5.1 CONCLUSION

The objective of the study, which is the optimization of bacterial cellulose yield by using RSM, was accomplished through the experimental model designed by CCD. The key of the success is mainly contributed by the type of variables selected and the minimum range of optimum value selected from the OFAT experiments prior experimental model design.

Based on the minimum range of the optimum value and the experiments data, RSM was successfully determine the significance variables that affect the fermentation process through statistical analysis. Since the variables of temperature, pH and glucose concentration was significantly affected the yield of bacterial cellulose, further application of specific optimum value for each variables in the fermentation process was enhanced the yield of bacterial cellulose 3.6 times than the conventional methods.

The specific optimum value of the variables was finalized as 29.2℃ for temperature, pH at 6 and 1.75 g/l of glucose concentration. Further analysis by FTIR and SEM has showed that the bacterial cellulose contained the typical spectrum and fibre network of the cellulose.

5.2 RECOMMENDATIONS

There are many factors related to the fermentation of bacterial cellulose, such as the type of carbon sources, the cultivation technique, the cultivation time, the dissolved oxygen rate and etc. These factors is believed to have own contribution to the yield of bacterial cellulose, respectively. Nevertheless, there are lack of researches has been conducted regarding to these factor and remain questionable.

Thus, further researches on the others factor by using RSM or any other new technology is suggested. Despite of that, further researches on the application of bacterial cellulose in water treatment or membrane-separation process is believed to have potential in revealing hidden benefits of the porous structure owned by bacterial cellulose membrane.

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APPENDIX A

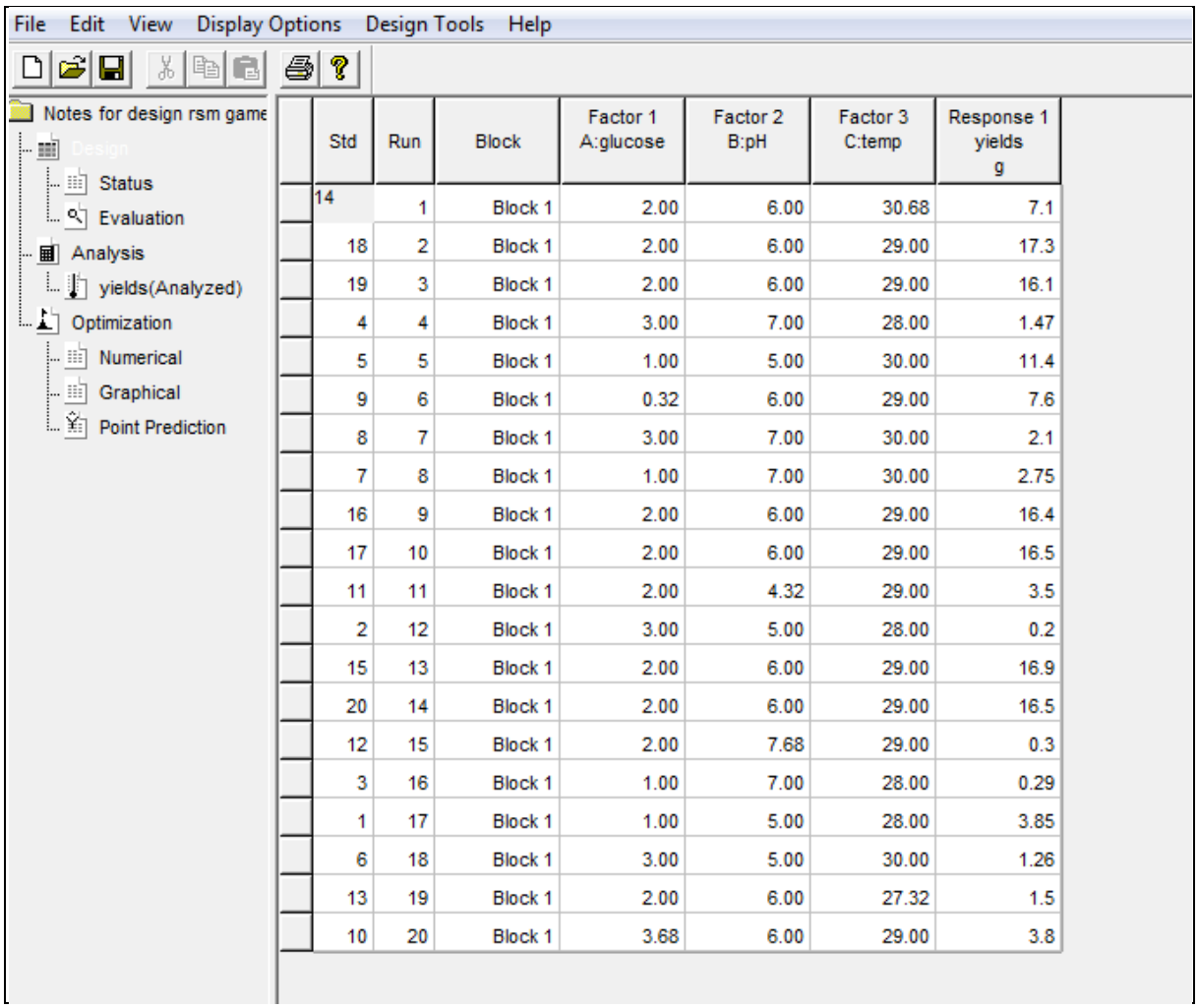
Images of FTIR Spectrophotometer

APPENDIX B

Images of SEM Equipment

APPENDIX C

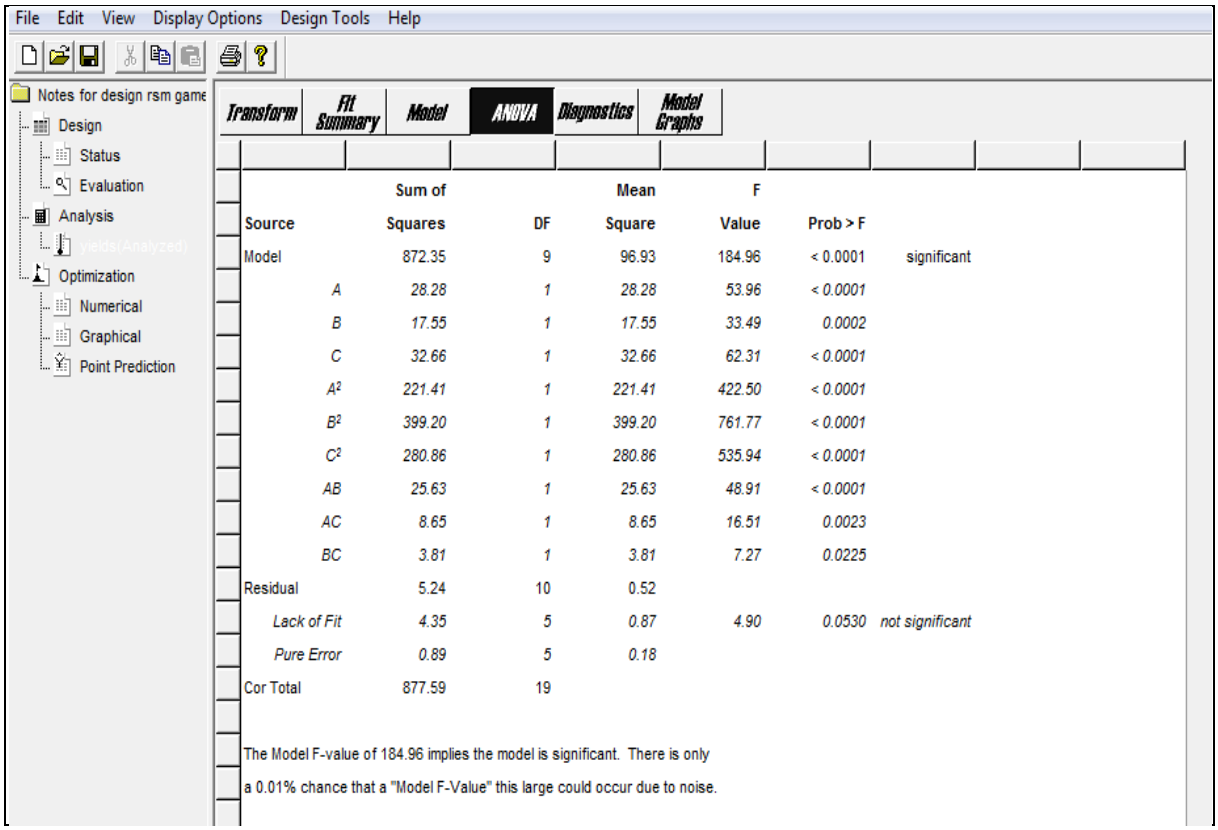
Experimental Model Designed by CCD



Std	Run	Block	Factor 1 A:glucose	Factor 2 B:pH	Factor 3 C:temp	Response 1 yields g
14	1	Block 1	2.00	6.00	30.68	7.1
18	2	Block 1	2.00	6.00	29.00	17.3
19	3	Block 1	2.00	6.00	29.00	16.1
4	4	Block 1	3.00	7.00	28.00	1.47
5	5	Block 1	1.00	5.00	30.00	11.4
9	6	Block 1	0.32	6.00	29.00	7.6
8	7	Block 1	3.00	7.00	30.00	2.1
7	8	Block 1	1.00	7.00	30.00	2.75
16	9	Block 1	2.00	6.00	29.00	16.4
17	10	Block 1	2.00	6.00	29.00	16.5
11	11	Block 1	2.00	4.32	29.00	3.5
2	12	Block 1	3.00	5.00	28.00	0.2
15	13	Block 1	2.00	6.00	29.00	16.9
20	14	Block 1	2.00	6.00	29.00	16.5
12	15	Block 1	2.00	7.68	29.00	0.3
3	16	Block 1	1.00	7.00	28.00	0.29
1	17	Block 1	1.00	5.00	28.00	3.85
6	18	Block 1	3.00	5.00	30.00	1.26
13	19	Block 1	2.00	6.00	27.32	1.5
10	20	Block 1	3.68	6.00	29.00	3.8

APPENDIX D

ANOVA Result of the Study



Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	872.35	9	96.93	184.96	< 0.0001	significant
A	*28.28*	*1*	*28.28*	*53.96*	*< 0.0001*	
B	*17.55*	*1*	*17.55*	*33.49*	*0.0002*	
C	*32.66*	*1*	*32.66*	*62.31*	*< 0.0001*	
A^2	*221.41*	*1*	*221.41*	*422.50*	*< 0.0001*	
B^2	*399.20*	*1*	*399.20*	*761.77*	*< 0.0001*	
C^2	*280.86*	*1*	*280.86*	*535.94*	*< 0.0001*	
AB	*25.63*	*1*	*25.63*	*48.91*	*< 0.0001*	
AC	*8.65*	*1*	*8.65*	*16.51*	*0.0023*	
BC	*3.81*	*1*	*3.81*	*7.27*	*0.0225*	
Residual	5.24	10	0.52			
Lack of Fit	*4.35*	*5*	*0.87*	*4.90*	*0.0530*	*not significant*
Pure Error	*0.89*	*5*	*0.18*			
Cor Total	877.59	19				

The Model F-value of 184.96 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Std. Dev.	0.72	R-Squared	0.9940
Mean	7.34	Adj R-Squared	0.9887
C.V.	9.86	Pred R-Squared	0.9560
PRESS	38.60	Adeq Precision	33.169

The "Pred R-Squared" of 0.9560 is in reasonable agreement with the "Adj R-Squared" of 0.9887.

APPENDIX E

Table of Optimization

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
glucose	is in range	1	3	1	1	3
pH	is in range	5	7	1	1	3
temp	is in range	28	30	1	1	3
yields	maximize	0.2	17.3	1	1	3

Solutions

Number	glucose	pH	temp	yields	Desirability	
1	1.75	5.84	29.22	17.067	0.986	Selected

1 Solutions found

Number of Starting Points 10

glucose	pH	temp
2.07	6.20	28.09
1.41	5.95	28.54
1.12	6.18	28.93
1.99	6.63	29.96
2.15	6.34	29.75
2.80	5.07	28.20
2.46	5.22	28.74
1.62	5.10	28.95
2.79	5.35	28.62
2.34	6.40	29.00

APPENDIX F

Calculation of Bacterial Cellulose Moisture Content

$$\text{Moisture} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100\%$$

$$= \frac{17.81 - 0.562}{17.81} \times 100\%$$

$$= 96.85\%$$

APPENDIX G

Calculation of Percentage Error between Predicted and Actual Yields

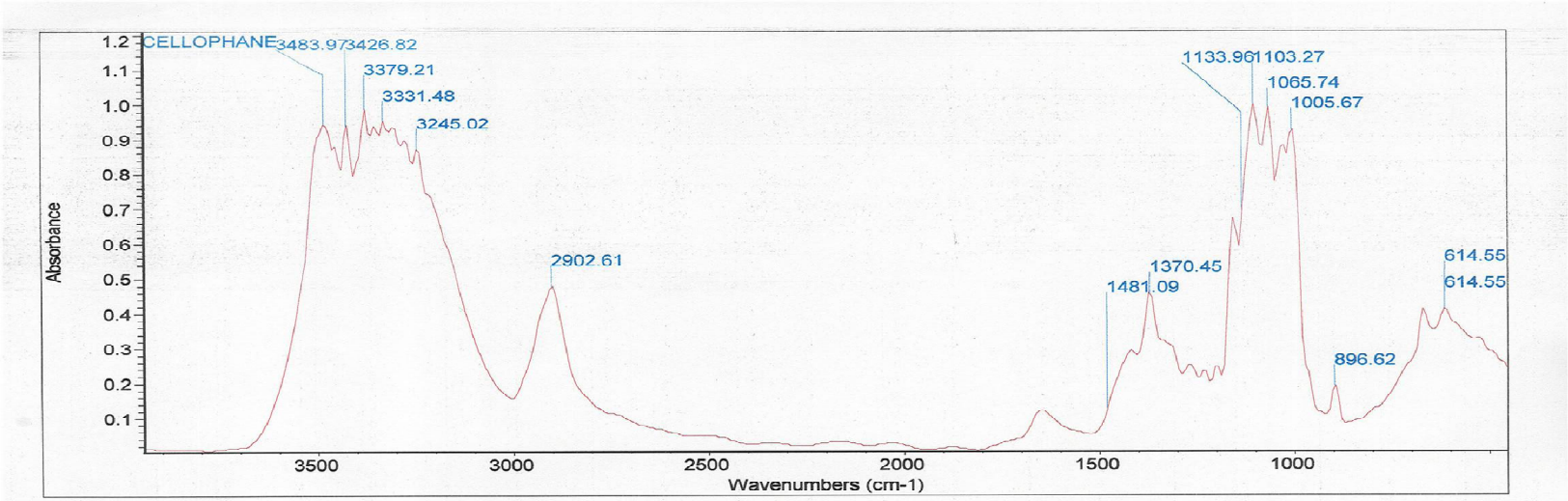
$$\text{Error} = \frac{\text{Actual} - \text{Predicted}}{\text{Actual}} \times 100\%$$

$$= \frac{17.81 - 17.067}{17.81} \times 100\%$$

$$= 4.17\%$$

APPENDIX H

FTIR Spectrum of Cellulose in Bacterial Cellulose



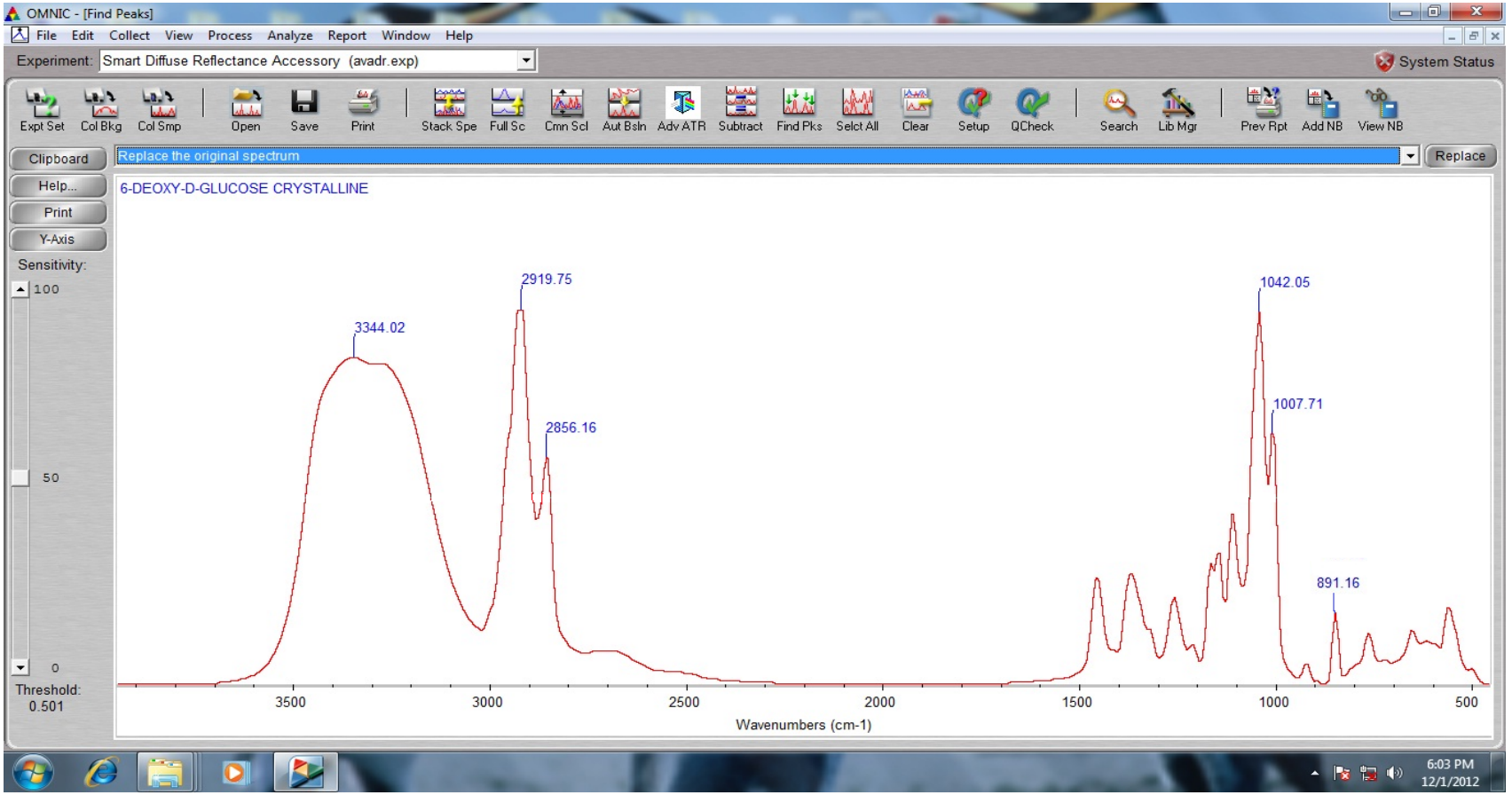
Tue Nov 29 16:35:49 2011 (GMT+08:00)
FIND PEAKS:

Spectrum:	CELLOPHANE	
Region:	3949.50	455.12
Absolute threshold:	0.007	
Sensitivity:	50	
Peak list:		

Position:	614.55	Intensity:	0.414
Position:	614.55	Intensity:	0.414
Position:	896.62	Intensity:	0.195
Position:	1005.67	Intensity:	0.933
Position:	1065.74	Intensity:	0.995
Position:	1103.27	Intensity:	1.001
Position:	1133.96	Intensity:	0.676

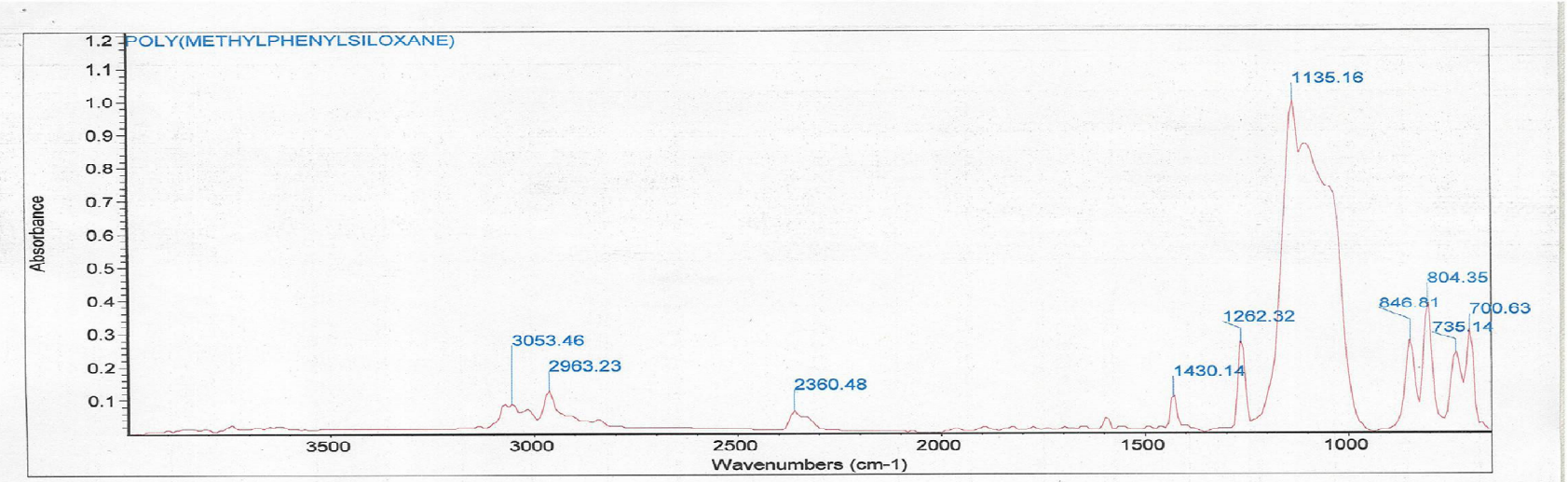
APPENDIX I

FTIR of Deoxyglucose in Bacterial Cellulose



APPENDIX J

FTIR Spectrum of PMPS in Bacterial Cellulose



Tue Nov 29 16:42:29 2011 (GMT+08:00)
FIND PEAKS:
Spectrum: POLY(METHYLPHENYLSILOXANE)
Region: 3999.84 649.93
Absolute threshold: 0.008
Sensitivity: 50
Peak list:

Position:	700.63	Intensity:	0.313
Position:	735.14	Intensity:	0.243
Position:	804.35	Intensity:	0.396
Position:	846.81	Intensity:	0.282
Position:	1135.16	Intensity:	1.003
Position:	1262.32	Intensity:	0.286
Position:	1430.14	Intensity:	0.117