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

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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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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).

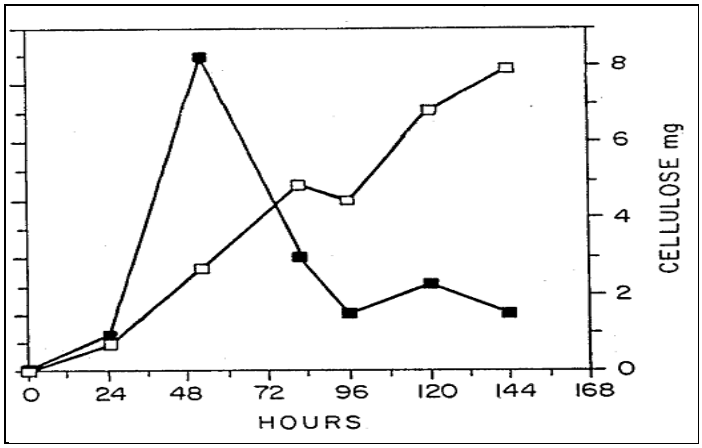

Figure 2.1: Cultivation Time of Bacterial Cellulose.

Source: Saxena et al. (2000)

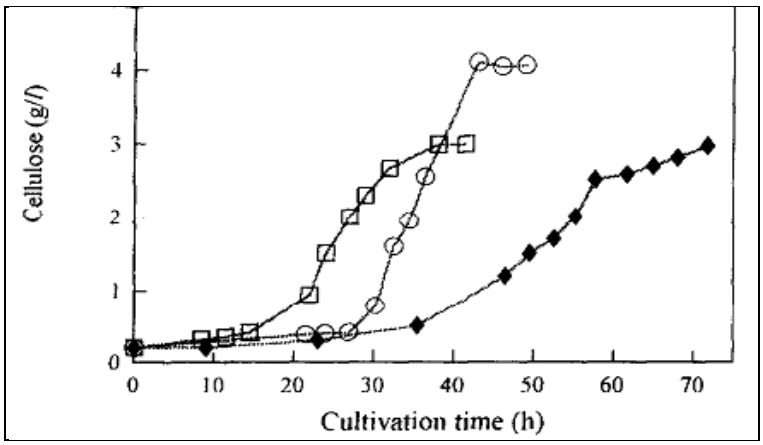

Figure 2.2: Cultivation Time of Bacterial Cellulose

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