PRODUCTION OF BACTERIAL CELLULOSE: EFFECT OF DIFFERENT MEDIUMS AND MODES OF OPERATION

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Bacterial cellulose, an exopolysaccharide produced by *Acetobacter Xylinum s.p.*, has unique structural and mechanical properties and is highly pure as compared to plant cellulose. This objective of this study was to assess the effect of different medium (Hestrin Schramm medium, banana peel, coconut water, and hibiscus waste) on microbial decomposition using three categories of operation mode (Batch, fed-batch and repeated batch). Production of bacterial cellulose in this experiment include the production of inoculum using the fresh coconut water and it was conduct under optimum temperature and pH which is 30°C and 5.5 respectively. From the result, banana peel gives the highest yield in fed batch mode of operation, followed by coconut water, hibiscus waste and Hestrin Schramm medium. The high production efficiency of banana peel was due to the high purity of carbon source in banana peel and the continuously feeding of the fermentation medium using the fed batch mode. These results show that, the consumption of carbon source is not the only factor that effects the efficiency production of bacterial cellulose. The operation mode might play another important factor in the production of bacterial cellulose. From the FTIR analysis, the bacterial cellulose produce appears to have very good crystallinity characteristics similar to the microcrystalline cellulose (MCC). SEM analysis concludes that the ribbon network is produce under the surface of bacterial cellulose.
ABSTRAK

Bakteria selulosa merupakan sejenis polisakarida yang dihasilkan daripada metabolism bakteria *Acetobacter Xylinum*. Bakteria selulosa memiliki struktur yang unik, ciri mekanikal yang teguh dan sangat asli jika dibandingkan dengan selulosa dari pokok. Objektif kajian ini adalah untuk mengkaji kesan medium yang berbeza ke atas pertumbuhan selulosa yang dihasilkan dari metabolism bakteria *Acetobacter Xylinum*. Medium yang digunakan adalah Hestrin Schramm, kulit pisang, air kelapa tua dan sisa bunga raya. Selain itu, objektif kajian ini juga adalah untuk mengkaji teknik fermentasi yang terbaik dalam penghasilan selulosa. Teknik fermentasi yang digunakan adalah fermentasi secara statik, fermentasi secara berterusan dan juga fermentasi secara berulang. Penghasilan bakteria selulosa dalam kajian ini termasuk penyediaan inokulum dengan menggunakan air kelapa sebagai medium fermentasi. Inokulum dan fermentasi bakteria selulosa dijalankan pada suhu dan pH optimum iaitu 30 °C dan pH 5.5. Dari hasil kajian, medium kulit pisang dalam teknik fermentasi berterusan menghasilkan kadar hasil selulosa yang paling tinggi diikuti medium dari air kelapa tua, sisa bunga raya dan Hestrin Schramm medium. Ini adalah kerana kandungan karbon yang tinggi dalam kulit pisang membolehkan selulosa dihasilkan dalam kuantiti yang banyak jika dibandingkan dengan medium yang lain. Dari hasil kajian ini juga membuktikan, teknik fermentasi juga mempengaruhi kadar penghasilan selulosa. Daripada analisis menggunakan FTIR, selulosa yang terbentuk mempunyai ciri Kristal yang sama dengan MCC. Keputusan dari SEM menunjukkan terdapat jalur seperti ribbon pada permukaan bakteria selulosa.
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LIST OF SYMBOLS

%  Percentage
°C  Degree Celcius
g  Gram
mL  Milliliter
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<td>CO₂</td>
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<td>FTIR</td>
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<td>KOH</td>
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<tr>
<td>K₂CO₃</td>
<td>Potassium Carbonate</td>
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<td>MARDI</td>
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<td>MCC</td>
<td>Microcrystalline cellulose</td>
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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Cellulose is the earth's major biopolymer and it is a tremendous economic importance globally (Delmer and Amor, 1995). Cellulose is the major constituent of cotton and wood. Together, cotton and wood are the major resources for all cellulose products such as paper, textiles, construction materials, cardboard, as well as such cellulose derivatives as cellophane, rayon, and cellulose acetate. The production of cellulose nowadays was very limited because we are using the plant to get this material, so it will affect the earth and also will affect the global warming condition (Frenchen, 2000). Preservation of forest resources is essential to prevent global warming because the increasing in CO₂ concentration can be stopped only by the absorption of CO₂ by plants and trees (Park et al., 1996). However, the use of trees for the production of paper and construction materials has continuously depleted forest resources (Nobles et al., 2001).

Bacterial cellulose is the only alternative for plant cellulose because bacteria produce bacterial cellulose in a few days, while trees need more than 30 years to realize full growth. In this respect, bacterial cellulose is one of the key material for preventing global warming and preservation of the nature (Steven, 2004). Several general of bacterium were used to produce the cellulose include Sarcina,
Agrobacterium, Rhizobium, and Acetobacter (Barbara et al., 2008) However, only Acetobacter Xylinum is only species known to be capable of producing cellulose (Brown, 1993). This organism and its product were first identified and characterized over a century ago, although both are very common in the production of vinegar industry. Acetobacter Xylinum is a gram negative bacterium and is unique in its prolific synthesis of cellulose (Brown, 1994). Acetobacter is the model system for study of the enzymes and genes involved in cellulose biosynthesis and also known as a new biopolymer. It has several unique properties such as high purity and ultrafine or fiber network, very high hydrophilic, good mechanical strength, and outstanding shape and retention and high crystalinity. The optimal temperature for growth is between 25° C to 30° C and the pH optimum about 5.4 to 6.3 (Vershuren, 1999).

To date, the process for production of bacterial cellulose commonly in the static cultivation method, with pellicles of bacterial cellulose formed on the surface of the static culture. However, this requires a large area in which to place the culture vessel and is impractical for large scale bacterial cellulose production (Okiyama et al., 1992). Therefore, an economical mass production system based on shaking culture condition is necessary. Bacterial cellulose produced by Acetobacter Xylinum has several unique properties include high polymerization, high purity and have a quality fiber network. One of the famous applications of bacterial cellulose is in the production of nata de Coco in Philippines which was the famous diet for Philippines dessert (Yoshinaga, 1997).

Fed-batch, repeated and batch culture are the mode using in this research. These three different modes are used to optimize the production of bacterial cellulose. Batch culture is a static where fermentation medium is added to a culture containing the substrate consisting of a carbon source, an energy source and nutrients. The cells grow using these and at some time later, one of these is exhausted. The carbon source that use in this research is glucose. If the carbon and energy source runs out, then the biomass stop growing and the remaining excess
nutrients are not up taken (Benziman, 1991). If the nutrient runs out first, then the remaining excess carbon and energy source may continue to be utilized. Thus further products may be formed. Since there are no nutrients remaining, there can be no further biomass (Tang et al., 2009). The second mode that to be optimized is repeated batch. Repeated batch is where the fermentation medium are added to culture the medium in a schedule time recycle, so the production of biomass is growth depend on the fermentation medium and it will give the effect to the concentration of biomass. In the repeated batch, the fermentation medium is added step by step until it arrive the optimum stage where the bacteria will produce high yield of concentration of bacterial cellulose (Park et al., 2001). The fed-batch mode of operation is where the fermentation medium is added to the culture continuously. This is to maximize the use of fermentation medium by bacteria to get the high production of bacterial cellulose (Okiyama, 2004). The composition in the fermentation medium also effects the production of cellulose. In adequate nutrient in fermentation medium will lead to the less productivity of bacterial cellulose. According to Brown et al. (1993), the carbon sources are very important to enhance the bacterial cellulose productivity. The carbon sources usually derived from the glucose or carbohydrate contents in the medium. If the fermentation medium possess a high number of glucose or carbon content, it will stimulate the bacteria to enhance the productivity.

1.2 PROBLEM STATEMENT

Until recently, much effort has been put on developing process for the production of bacterial cellulose. The amount of biomass obtain in the reactor is comparatively low and therefore exist a need for an improved process for the production of bacterial cellulose. For the previous report, the production of bacterial cellulose was very low in the agitated culture (Tsuchida et al., 1997). So in this study, the method of static culture is used to see the production of bacterial cellulose. A wide variety of modes were found to have ability to produce the high concentration of biomass in the production of bacterial cellulose. However, the
problem exists was to select the mode that not only can produce the bacterial cellulose but at the same time must have the highest biomass and substrate consumption (Okiyama et al., 2003). Furthermore, an economical source is essential to have successful operation of the process. The main reason of this study is because most the production of cellulose is based on the plant cellulose. When the plant is cut everyday just to get the cellulose, it will give the effect to the global warming (French, 2007). This study will provide other alternative in production of cellulose. The last problem about the cellulose is in the plant cellulose itself. The production of plant cellulose will take a lot of time (Park et al., 2000). For example, the plant or tree will take about several months to grow, instead of this matter the production of bacterial cellulose will only take about a week for the production of cellulose.

1.3 RESEARCH OBJECTIVE

The objectives of this research is to determine the production of bacterial cellulose by Acetobacter Xylinum s.p in a static culture using different mediums and modes of operation which are batch, fed batch and the repeated batch using glucose as the carbon source

1.4 SCOPE OF STUDY

1.4.1 To determine modes of operation that can produce highest yield of bacterial cellulose which are batch, fed batch or repeated batch by using the glucose as a carbon source

1.4.2 To determine the medium that can produce high yield of bacterial cellulose

1.4.3 To study the characteristic bacterial cellulose using the FTIR

1.4.4 To characterize the morphology of the BC produced by using Scanning Electron Microscope
1.5 SIGNIFICANCE OF THE RESEARCH

There are several significant of this study that can be review from previous research paper. The most important thing that the production of bacterial cellulose can reduce the global warming in the earth (Brown, 1989). This is very obvious effect due to the reason that decreases of dependability to the plant cellulose. Besides that, the purpose of this study is, to increase the production of bacterial cellulose in large scale. In this study, the production of bacterial cellulose is conduct in three different modes. One of the modes will give the high yield of production and this mode will be used to increase the production of bacterial cellulose in future. In fact, the significant of this study is to decrease the dependability to the plant cellulose (Brown, 1989). This study is the most appropriate alternative that can be implementing in production of bacterial cellulose.
CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Cellulose is the most abundant organic polymer in nature, where it plays the important role in the plant cell (Delmer and Amor, 1995). Plant is used widely in the production of paper and textile industries. It is also important to know that the plant also play a very important role in the preventing the global warming from becoming worst. So the scientist and researcher have come with a new alternative to get new cellulose. The bacterial cellulose is the one of the alternative that was used widely in this world. The first man who study and discovered about the bacterial cellulose was Adrian Brown in 1886 (Brown, 1986). Bacterial cellulose is extremely pure and exhibit high degree of polymerization. It is known that some bacteria will produce the cellulose by their metabolism system. In addition, there are some strains of the non-photosynthetic organism *Acetobacter* which have the ability to synthesize high quality cellulose (Brown et al., 1992). Bacterial cellulose has the good and quality cellulose which make them very useful.

2.2 APPLICATION OF BACTERIAL CELLULOSE

2.2.1 Food Application

Cellulose that has a high purity in chemical properties has been used in processed foods as thickening and stabilizing agent (Iguchi, 2005). The first use of
bacterial cellulose in food industry was in the production of nata de coco in the Philippines (Bhudiono, 2005). The gel-like properties of bacterial cellulose, combined with its complete indigestibility in the human intestinal tract, made this an attractive food base (Rosidi, 1999). In 1970s, mevinolin which is the important metabolite in Monascus sp, has been identified and shown to inhibit the production of cholesterol (Westland et al., 1990). Nata is one of the bacterial cellulose products. Nata is prepared from the metabolism of Acetobacter Xylinum is a popular food in the Philippines and Asia countries. It also use widely in food processing because it contain a soft texture and high fiber. The combination of functional characteristics of monacolin K and bacterial cellulose in Monascus – nata complex have potential novel functional food stuff (Yamamoto, 2006). In 1992s, bacterial cellulose has been use for production of diet stuff that been introduce in the drink. Acetobacter was grown along with the yeast and the tea extract and sugar. This is consumed as a kombucha, or Manchurian tea for improved health needs (Stephens, 1990)

2.2.2 Pharmaceutical and medical application

Bacterial cellulose contains high tensile strength, high porosity and microfibrillar structure (Brown, 1999). For example, the chronic wounds such as venous and diabetic ulcer are very difficult to heal. The treatment for the chronic wounds involves a lot of materials. As bacterial cellulose contain of highly porous material, it allow the potential transfer antibiotics or other medicine into the wound (Takagi, 1993). It was report by Bielecki to satisfy the requirements of modern wound dressing material. Bacterial cellulose has a very high water holding capacity (Ichimura et al., 1998). This characteristic made the bacterial cellulose is very effective to heal the ulcers and wounds. Brazilian company Biofil has investigate the unique characteristics that can heal the wound and they produce two product which is Bioprocess and Gengflix that acts as a dressing material for extensive wounds (Fontana et al., 1990, 1991). A bacterial cellulose produce by Xylos Corp (USA) namely as Prima Cel™ has been applied in clinical test for heal the ulcer and wounds (Risberg et al., 2006). Tissue engineered blood vessel (TEBV) represent an
alternative for overcome reconstructive problem that have a link with the vascular disease by providing small caliber vascular grafts and bacterial cellulose exhibits the properties that can effectively use as a scaffolds for tissue engineering blood vessels (Helenius et al., 2006)

2.2.3 Other application

The unique physical and mechanical properties of bacterial cellulose such as high reflectivity, flexibility, light mass and ease of portability has been apply in the electronic paper display (Shah et al., 2005). The bacterial cellulose fragmented has very good prospects in papermaking which are very suitable for bank note and also the bible paper (Iguchi et al., 2000). Bacterial cellulose also has been applied in the mineral and oil industry. The new research from the University of Michigan reported by Westland in 2009, there are pattern inventions that have a relation to the use of bacterial cellulose in hydraulic fracturing of geological formations at selected levels of wells drilled for recovery of hydrocarbon. Addition of cellulose microfibrils obtained by acid hydrolysis of cellulose fibers at low concentration to polymer gels and films has shown the significant change in the strength and mechanical properties (Laszkiewicz, 1876). Based on the tensile strength, the bacterial cellulose has a great potential to be used as a packaging material in food packaging where the continuous moisture removal and minimal oxygen transmission properties play an important role (Jonas and Farah, 1995). The unique dimensional stability of bacterial cellulose give a potential in making the sound transducing membrane which can maintains the high sonic velocity over a many frequencies ranges. Thus, the Sony Corporation in Japan has taken this advantage to develop the first audio speaker diaphragms using bacterial cellulose (Ichimura et al., 1988)
2.3 BACTERIUM STRAIN

Among the bacteria, one of the most advances types of purple bacteria is the common vinegar bacteria that are *Acetobacter Xylinum* (Brown, 1986). The *Acetobacter* is a non-photosynthetic organism that can use the glucose, sugar, and glycerol and turn it to the pure cellulose (Brown *et al*., 1976). A typical single cell of *Acetobacter Xylinum* can convert about 108 glucose molecules per hours to cellulose (Brown *et al*., 1989). A single cell of *Acetobacter Xylinum* has a linear row of pores from which glucan chain polymer aggregates are spun. *Acetobacter Xylinum* have a great absorptivity strength constitute two of many novel features of bacterial cellulose (Brown, 1989; White and Brown, 1989; Brown, 1992; Brown, 1994). The optimal temperature for growth of *Acetobacter Xylinum* between 25°C to 30°C and the pH optimum is about 5.4 to 6.3 (Verschuren, 1999). *Acetobacter Xylinum* has been applied as a model of microorganism for basic and applied in the production of bacterial cellulose (Park *et al*., 2003). It is commonly has been used in the study because it possess the ability to produce high level of polymer from a wide range of carbon and nitrogen sources (Delmer *et al*., 1991, Weigend *et al*., 2007). It is a gram negative that have a rod shape bacteria that produce the cellulose in the form of interwoven extracellular ribbons as part of primary metabolite. This bacterium grows and produces cellulose from a wide range variety of substrate. The Table 2.1 shows the different strain that produce the bacterial cellulose.
Table 2.1: Strain for production of bacterial cellulose

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Carbon Sources</th>
<th>Supplement</th>
<th>Culture Time</th>
<th>Yield (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. xylinum</em> BRC 5</td>
<td>Glucose</td>
<td>Ethanol, Oxygen</td>
<td>50h</td>
<td>15.30</td>
</tr>
<tr>
<td><em>G. hansenii</em> PJK (KCTC 10505 BP)</td>
<td>Glucose</td>
<td>Oxygen</td>
<td>48h</td>
<td>1.72</td>
</tr>
<tr>
<td><em>G. hansenii</em> PJK (KCTC 10505 BP)</td>
<td>Glucose</td>
<td>Ethanol</td>
<td>72h</td>
<td>2.50</td>
</tr>
<tr>
<td><em>Acetobacter</em> sp. V6</td>
<td>Glucose</td>
<td>Ethanol</td>
<td>8 day</td>
<td>4.16</td>
</tr>
<tr>
<td><em>Acetobacter</em> sp. A9</td>
<td>Glucose</td>
<td>Ethanol</td>
<td>8 day</td>
<td>15.20</td>
</tr>
<tr>
<td><em>A. xylinum</em> BPR 2001</td>
<td>Molasses</td>
<td>None</td>
<td>8 day</td>
<td>7.820</td>
</tr>
<tr>
<td><em>A. xylinum</em> BPR 2001</td>
<td>Fructose</td>
<td>Agar oxygen</td>
<td>72h</td>
<td>14.10</td>
</tr>
<tr>
<td><em>A. xylinum</em> BPR 2001</td>
<td>Fructose</td>
<td>Agar</td>
<td>56h</td>
<td>12.00</td>
</tr>
<tr>
<td><em>Acetobacter xylinum ssp.sucofermentans</em> BPR2001</td>
<td>Fructose</td>
<td>Oxygen</td>
<td>52h</td>
<td>10.40</td>
</tr>
<tr>
<td><em>Acetobacter xylinum ssp.sucofermentans</em> BPR2001</td>
<td>Fructose</td>
<td>Agar oxygen</td>
<td>44h</td>
<td>8.70</td>
</tr>
<tr>
<td><em>Acetobacter xylinum</em> E25</td>
<td>Glucose</td>
<td>No</td>
<td>7 day</td>
<td>3.50</td>
</tr>
<tr>
<td><em>G. xylinus</em> strain (K3)</td>
<td>Mannitol</td>
<td>Green tea</td>
<td>7 day</td>
<td>3.34</td>
</tr>
<tr>
<td><em>Gluconacetobacter xylinus</em> IFO 13773</td>
<td>Glucose</td>
<td>Lignosulphonate</td>
<td>7 day</td>
<td>10.10</td>
</tr>
<tr>
<td><em>Acetobacter xylinum</em> NUST4.1</td>
<td>Glucose</td>
<td>Sodium alginate</td>
<td>5 day</td>
<td>6.00</td>
</tr>
<tr>
<td><em>Gluconacetobacter xylinus</em> IFO 13773</td>
<td>Molasses</td>
<td>No</td>
<td>7 day</td>
<td>5.76</td>
</tr>
<tr>
<td><em>Gluconacetobacter</em> sp. RKYs+</td>
<td>Glycerol</td>
<td>No</td>
<td>144h</td>
<td>5.63</td>
</tr>
</tbody>
</table>

Sources: Son *et al.* (2001)
2.4 BACTERIAL CELLULOSE BIOSYNTHETIC PATHWAY

Synthesis of bacterial cellulose involves multiple step process and a large number of enzymes. The process includes the formation of uridine disphoglucose (UDPGlc) which is the precursor in the cellulose formation (Brown et al., 1987). Then, its follow by glucose polymerization into the β-1-4 glucan chain and a chain is form like a ribbon structure of cellulose chain. Its form in a hundred or even a thousand of individual cellulose chain (Delmer et al., 1995). In Acetobacter Xylinum, cellulose synthesis is tightly connected with the catabolic process of oxidation and use approximately 10% of energy derived from the catabolic reactions (Amor et al., 1995). Acetobacter Xylinum converts various carbon compounds such as hexoses, glycerol, glucose and molasses into cellulose (Bielecki et al., 2005). Figure 2.1 shows the biochemical pathway for cellulose synthesis by Acetobacter Xylinum.

The synthesis of cellulose in Acetobacter Xylinum or any other cellulose follows two steps. First is formation of β-1-4 glucan chain polymerization of glucose units and the second is assembly and crystallization of cellulose chain (Lin et al., 1985). The rate of polymerization is limited by the rate of assembly and crystallization. Cellulose synthase catalyzes the cellulose biosynthesis by polymerizing the glucose units into the β-1-4 glucan chain (Valla et al., 1989). The formation of cellulose fibrils is shown in Figure 2.2.

Two hypotheses for this mechanism in Acetobacter Xylinum have been reported. The first hypothesis assumes that the polymerization of the β-1-4 glucan chain does not involve a lipid intermediate (Delmer et al., 2000). The glucose residue were added to the nonreducing end of the polysaccharide and those reducing ends were nascent polymer chains situated away from the cells (Yamanaka et al., 1993). The second hypothesis states that the polymerization of β-1-4 glucan involves a lipid intermediate (Benziman et al., 1989). The involve of
lipid intermediate in the synthesis of acetan which is a soluble polysaccharide has been proven. The polymer synthesis produce no lipid component (Klemm, 2003)

**Figure 2.1:** Biochemical pathway for cellulose synthesis by *Acetobacter Xylinum.*

CS cellulose synthesis, GK glucokinase, FBP fructose-1,6-bi-phosphate phosphatase, FK fructokinase, 1FK fructose-1-phosphate kinase, PGI phosphoglucoisomerase, PMG phosphoglucumutase, PTS systems of phosphotransferases, UGP pyrophosphorylase uridine diphosphogluucose, UDPglc uridiene diphosphosglucose, G6PDH glucose-6-phosphate dehydrogenase, NAD nicotinamide adenine dinucleotide, NADP nicotinamide adenine dinucleotide phosphate

Sources: Cooper *et al.* (1985), Saxena *et al.* (2000)
2.5 PROPERTIES OF BACTERIAL CELLULOSE

Bacterial cellulose possesses a very high crystallinity, high tensile strength, extremely insolubility in most of the solvents, moldability and high degree of polymerization (Ramana et al., 2005). The thickness of cellulose fibrils is commonly 0.1-10μm, one hundred times thinner than cellulose fibrils obtained from the real plant (White et al., 1981). The water holding capacity is 100 times higher (Schrecker et al., 2005). The bacterial cellulose is far stronger than plant cellulose (George et al., 2005). The macroscopic morphology of cellulose is depending on the condition of the culture which can easily be tailored for the physicochemical properties (Wanichapichart et al., 2005). Cellulose is soluble in concentrated acids like sulphuric, hydrochloric or nitric acid. It also soluble in 8.5% NaOH solution. The solubility of cellulose in the alkali can be increases by adding 1% of urea to the solution (Sabapathy et al., 2005).