

EFFECT OF CULTURE CONDITIONS ON BACTERIAL CELLULOSE (BC) PRODUCTION FROM *ACETOBACTER XYLINUM* TISTR976 AND PHYSICAL PROPERTIES OF BC PARCHMENT PAPER

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Abstract

In this study, *Acetobacter xylinum* strain TISTR976 was cultured in coconut juice base medium under three culture conditions: static tray, shaking flask, and a 5 liter stirring bioreactor, with optimal pH 4.5 at 30°C. From these three culturing conditions, BC yields of 4.73, 5.32, and 7.94 g/liter were obtained on day 6 of cultivation. By using a specific process, the BC from the three conditions was made into paper called BC paper. The BC papers produced from three culture conditions were tested for physical properties, and studied for micro-structures by a scanning electron microscope (SEM). The physical properties of the BC papers, such as wet strength retention were figured out as 124.2, 25.7, and 12.2%, respectively, however, the oil resistant value of the bioreactor BC paper was so poor in comparison with that of the static tray and shaking flask. From this evidence, it was shown that only BC paper from the static tray and shaking flask exhibited the characteristics of specialty parchment paper. Furthermore, the SEM micrographs of BC paper from the static tray and shaking flask showed the crystalline fibril layers with an orderly fibril layer arrangement, whereas that from the bioreactor was disorderly. A hypothesis on the correlation between the parchment paper property and the orderly fibril layer arrangement of BC paper has been notified.

Keywords: *Acetobacter xylinum*, optimization, bacterial cellulose, parchment paper

Introduction

Acetobacter xylinum (*A. xylinum*) is a gram-negative bacterium, which can produce cellulose and acetic acid during growth and release them into the environment. This

cellulose has a common name, bacterial cellulose (BC). The BC is pure and its extra-cellular excretion can form aggregated fibrils which crystallize into ribbons and assemble

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into a thick cellulosic mat known as a pellicle. Because of its ultra-fine network structure, high biodegradability and unique mechanical strength (Brown, 1992), the BC is expected to be used for many industrial applications such as a food additive (Okiyama *et al.*, 1993), high-strength construction materials (Iguchi *et al.*, 2000), wound care products (Jonas and Farah, 1998), and paper.

Vegetable parchment is defined as a high quality paper product obtained by parchmentation with a waterleaf sheet that formed from purified cellulose fibers (Smook, 1990). The sheet is then surface-sized dried and calendered to the extent required for the finished product. Vegetable parchment is either greaseproof or grease resistant. It has high wet strength and is resistant to disintegration by any chemical agents. It is widely used in food packaging, printing parchment, and release paper. Papers of special properties, for example wet strength paper, must have a wet strength retention range from 25 to 30%. The high wet strength paper must have a wet strength retention value not less than 15%, while paper without a wet strength agent would have a wet strength retention range from only 3 to 10% (Casey, 1960).

We found in our previous work that paper made from BC derived from static condition showed the physical properties of a parchment paper (Suwannapinunt *et al.*, 2001). However, the static condition required 7 to 9 days of cultivation to get the BC sheet. Besides, oxygen is an important factor for BC production by *A. xylinum* (Watanabe and Yamanaka, 1995), we tried to vary the culturing methods that might give the higher BC yield in a short time. In this research, the *A. xylinum* TISTR976 was cultured under three conditions and the BC obtained from each culture condition was processed into paper called BC paper. Simultaneously, the physical and mechanical properties, as well as the microscopic structures observed by the SEM of the BC paper were characterized. The results suggested that BC paper could be applied by industry, for example as biodegradable packaging for the food industry, home decorating goods, etc.

Materials and Methods

Bacterial Strain and Culture Media

Acetobacter xylinum TISTR976 used in this study was obtained from the Thailand Institute of Science and Technology Research. The strain was cultured on glucose yeast extract (GYE) agar which contains the following constituents per liter: D-glucose 100 g, yeast extract 10 g, peptone 5 g, CaCO₃ 20 g, and agar 25 g. BC production was performed in liquid medium by using coconut juice as the base medium; 1 liter of medium contains coconut juice 1 liter, sugar cane 30 g, and (NH₄)₂SO₄ 3 g. The pH of the medium was adjusted with glacial acetic acid to 4.5. All media were autoclaved at 121°C for 15 min.

Preparation of *A. xylinum* TISTR976 as a Starter Culture

A. xylinum TISTR976 was cultured on GYE agar at 30°C for 5 days. Pre-inoculum of all experiments were prepared by transferring 1 colony of *A. xylinum* TISTR976 grown on GYE agar to a 250 ml Erlenmeyer flask containing 100 ml of coconut juice medium. After cultivation, the cellulose pellicle formed on the surface of the culture broth was vigorously shaken to remove active cells embedded in the cellulose membrane and then filtrated through gauze. The filtrate was then measured for optical density at 600 nm (OD₆₀₀) and was diluted to 0.3 (approx. 1.0×10^3 CFU/ml) and used as a seed culture (5% inoculum) in the experiments.

The strain was cultivated under three conditions, static culture (static tray), shaking condition (shaking flask), and agitated condition (5 liter stirring bioreactor, a product of B Braun Biotech International, Germany). In the static tray, 75 ml of seed culture was transferred onto a plastic tray having the size of 36 × 22 × 9 cm that was filled with 1.5 liters of sterile coconut juice medium. After inoculation, the tray was covered with sterile newspaper and left on a shelf at room temperature (27 - 30°C). In the shaking flask, 5 ml of seed culture was transferred into a 250 ml Erlenmeyer flask filled with 100 ml of sterile coconut juice medium, and the culture was aerated by shaking in a rotary

shaker at 50 rpm at 30°C. In the 5 liter stirring bioreactor, 150 ml of seed culture was transferred into the reactor vessel filled with 3 liters of sterile coconut juice medium. This culture was aerated by supplying filtered air at 6 liter/min (1.5 vvm) and setting the agitating speed at 200 rpm. The reactor temperature was maintained at 30°C and the pH was adjusted to 4.5 by automatic addition of 5 N NaOH and 1 N HCl solutions. BC was produced from each culture condition by using 3 liters of medium in a period of 6 days.

Determination of BC Production

After 6 days of cultivation, the BC was harvested and purified by treating with 0.5 N NaOH at 90°C for 1 h to remove the bacterial cells and medium components and then rinsed with water three times. The BC pellicle was dried at $105 \pm 2^\circ\text{C}$ for 12 - 24 h, or until at constant weight per gram.

Paper Production from BC

BC from the three culture conditions was harvested and neutralized by soaking in 1% (NH_4)OH for 24 h. Then the BC was boiled in deionized water for 30 min and divided into 3 groups according to the culture conditions. Each group was pressed with a 10 ton press unit to squeeze out water and to get the BC wet sheets. The BC wet sheets were bleached by soaking in 5 liters of 1.5% H_2O_2 , which was adjusted to pH 11 with 0.5% NaOH, in a sealed plastic bag. The plastic bag with the BC was then shaken for 10 min and put into boiling water for 30 min or until the BC color turned white. After bleaching, the white BC sheets were washed with tap water and pressed again with a 10 ton press unit; the wet white BC sheets were dried on an electrical press unit at a high temperature of 140°C for 30 sec. The dried white BC sheets were called BC paper and were tested for physical properties further.

Physical Property Testing of BC Paper

Before testing, the samples of BC paper (21×28 cm) were conditioned in a testing room having relative humidity (RH) of $65 \pm 2\%$ and a temperature of $27 \pm 1^\circ\text{C}$ for at least 4 h. The BC

paper was tested for physical and mechanical properties by following the methods in standards of the Technical Association of Pulp and Paper (TAPPI, 1998) to as follows;

- Basis weight (BW, g/m^2) was the grammage of paper, weight per unit area (TAPPI, T410 om-98).

- Thickness (mm) was tested by using a micrometer type caliper (TAPPI, T411 om-97).

- Tensile strength (TS, kgf/cm or kN/m) was the maximum tensile force per unit width developed in a test specimen at rupture or break under prescribed conditions. (TAPPI, T404 om-92).

- Tensile index (kN.m/kg) was the ratio of tensile strength divided by basis weight (T404 om-92).

- Wet tensile strength (wet TS, kgf/cm or kN/m) was the maximum tensile force per unit width developed in a wet test specimen at rupture or break. Especially, the test paper strip has to be soaked in distilled water for at least 4 h. After that, the excess water was removed by blotting on tissue paper and then the wet specimen was ready to be tested (TAPPI, T456 om-96).

- Wet strength retention (%) was the percentage on retention of the strength in comparison with wet and dry conditions of specialty paper, calculated by wet tensile strength divided by dry tensile strength and multiplied by 100 (Casey, 1960).

- Tearing strength (TA, mN) was the measurement of the force, perpendicular to the plane of the paper, required to tear multiple plies through a specified distance after the tear has been started using an Elemendorf-type tearing tester (TAPPI, T414 om-98).

- Tearing index ($\text{N.m}^2/\text{kg}$) was calculated from the tearing strength divided by the basis weight (TAPPI, T414 om-98).

- Porosity & air resistance of paper (sec/50 ml) was the time measurement in seconds on the air resistance of approximately 1 sq.in. of a circular area of paper using a pressure differential of 1.22 kPa. (TAPPI, T460 om-96).

- Castor oil penetration (min/0.05 ml) was the measurement of the time required for a drop

of castor oil to produce a uniform translucent spot on the underside of the test specimen by permeating through the specimen (TAPPI, T462 om-93).

- Translucence was the scrutinizing of light concentration quality that passed through the tested specimen laid on the top of a circular aperture in the platform of the equipment which had a light bulb set below. The light intensity was scored into 5 levels; dark = 0, light dark = 1, opaque = 2, lightly opaque = 3, translucent = 4.

Each test was performed twice (requiring 2 sheets of BC paper) and the mean values and standard deviations were calculated with high significance ($P < 0.05$) on consistency.

Microscopic Study of BC Paper by Using Scanning Electron Microscope (SEM)

Fixed and dehydrated BC papers were cut and then coated with gold. A Hitachi S-2500 field emission scanning electron microscope at 10 or 15 kV was used for sample examination. The pictures were captured both top view and side view with 500x magnification.

Results and Discussion

Effect of Culture Conditions on BC Production

The BC yield from the 5 liter stirring bioreactor was the highest (7.94 g/l), followed by that of the shaking flask (5.32 g/l), and the

lowest was that of the static tray (4.73 g/l), as shown in Figure 1. The three BC culture conditions were different in their aeration patterns; the 5 liter stirring bioreactor provided a good aeration and mixing, the shaking flask provided some aeration during shaking, whereas with the static tray the aeration occurred only at the surface of the liquid medium. Therefore, it could be said that the oxygen concentration is an important factor for BC production by *A. xylinum*. Watanabe and Yamanaka (1995) had reported that oxygen concentrations affected BC production, as 10% of oxygen concentrations could reach to a 25% higher yield of BC production without any influence on cell growth. Moreover, the report of Cannon *et al.* (1991) mentioned that the aeration pattern also affected the cell growth of *A. xylinum*. In their experiments, the doubling time for agitated culture was short, only 4 to 6 h, while that of the static culture was between 8 to 10 h. For the static culture, the cellulose formed at the surface air/liquid interface of the medium. Borzani and Souza (1995) had reported that the real site for BC formation was at the upper side of the pellicle, the air/pellicle interface. When the pellicle became thicker and thicker, the producing cells and the nutrition sources were more difficult to be transported through the pellicle up to the medium surface (Jonas and Farah, 1998), causing the slow formation

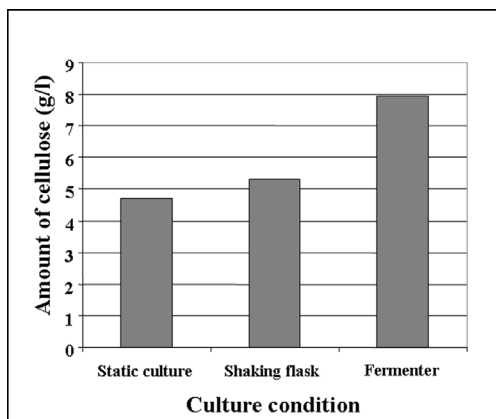
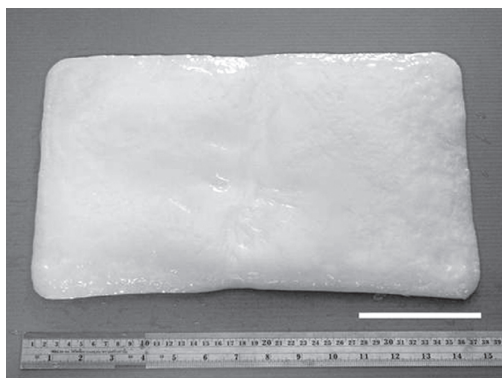


Figure 1. Amount of cellulose (g/l) produced by *A. xylinum* TISTR976 cultured under three conditions, static tray, shaking flask, and 5 liter stirring bioreactor

of BC. Besides, as oxygen is needed for BC formation, the static culture required a large surface area (Masaoka *et al.*, 1993). They analyzed a surface volume ratio (S/V ratio) of the BC production that ranged from 0.13 to 1, and found that the BC producing rate was dependent on the surface area. They calculated their BC strain at a producing rate of 2.4×10^{-3} g/day/cm². In this study, the S/V ratio was 0.53 and could achieve about 1×10^{-3} g/day/cm². Therefore, the medium volume used to produce BC with a desired thickness could be adjusted but the S/V ratio must be considered.

The Comparison of the Physical Properties of BC Papers Obtained from the Three Culture Conditions

The macro-morphology of the BC produced from the three culture conditions with different aeration patterns was found to be significantly different as shown in Figure 2. The BC from the static tray showed a thick pellicle with a shape like a paper sheet, while that of the shaking flask also showed a thick pellicle but with a round shape as the diameter of the 250 ml Erlenmeyer flask, and the BC from the 5 liter



(a)



(b)



(c)

Figure 2. BC produced from *A. xylinum* TISTR976 under static condition (a), shaking condition (b), and in 5 liter stirring bioreactor (c)

stirring bioreactor showed morphology that looked like smudge pulp membrane. Watanabe *et al.* (1998) had reported that the difference in macroscopic morphology of fresh BC between static and agitated cultures was caused by the various degrees of crystallinity, which depended strictly on the culture conditions. Furthermore, they reported that the agitated BC had a lower crystallinity index and a smaller crystalline than static BC.

From this research, there was much evidence from which it could be concluded that the different culture conditions of BC not only affected the BC yield and the BC macro-structure, but also affected the physical properties of the BC paper made from that BC. The physical property test results of the three conditions of recorded BC paper, as shown in Table 1, exhibited significant differences in physical properties of the BC paper from each culture condition. The static tray BC paper showed the highest Tearing index at 3.70 Nm²/kg, but those of the shaking flask and stirring bioreactor showed low Tearing indexes

at 1.38 and 0.73 Nm²/kg, respectively. Moreover, BC paper from the static tray showed the highest Wet strength retention value of 124.18%, as compared with those from the shaking flask and stirring bioreactor, which showed very low Wet strength retention values of 25.71 and 12.17%, respectively. Besides, the Castor oil penetration test (oil-grease proofness) showed that only BC paper from the static tray exhibited the highest oil resistance among the three BC culture conditions. By having a high Wet strength retention (of more than 15%) and high Castor oil penetration proof (of more than 24 h) for the BC paper from the static tray and shaking flask, it could be concluded that only these two culture conditions could be used to produce BC paper which had characteristics of a special property paper named parchment paper. The morphology of BC obtained from the 5 liter stirring bioreactor which was like pulp or a disintegrated form when it was processed to be paper exhibited low physical properties. However, BC paper obtained from all three culture conditions has the same special

Table 1. Physical property test result of gasket (commercial parchment paper) and papers obtained from BC produced by *A. xylinum* TISTR976 cultured in three conditions

Physical property of paper	Gasket (parchment paper) and papers produced from BC obtained from three different culture conditions			
	Gasket	Static tray	Shaking flask	Bioreactor
Basis weight (g/m ²)	586.9±3.60	105.78±7.5	139.70±5.23	151.84±11.40
Thickness (mm)	0.82±0.011	0.18±0.010	0.128±0.04	0.21±0.046
Tensile strength (kgf)	14.40±0.40	5.5±1.5	6.65±1.84	6.24±1.10
Tensile index (kN.m/kg)	16	34.00	31.28	26.87
Tearing strength/mN	5,729.42±226.9	392.4±19.6	192.93±5.6	111.18±22.65
Tearing index (Nm ² /kg)	9.8	3.70	1.38	0.73
Wet tensile strength (kgf)	8.36±0.12	6.83±2.7	1.71±1.24	0.76±0.44
Wet strength retention (%)	58.1	124.18	25.71	12.17
Castor oil penetration (min/0.05 ml)	18,918.0±4906.8	Oil resistant permanently	Oil resistant within 6 months	Oil resistant within 6 months
Air penetration test (sec/50 ml)	366.6±9.33	Air impenetrate	Air impenetrate	Air impenetrate

property of oil resistance, so they might be developed for application for the packaging industry.

Yamanaka *et al.* (1989) had reported that the native *Acetobacter* pellicle had mechanical properties including shape retention and tear resistance that were superior to many synthetic fibers. They also observed that BC displays a Young's modulus value (a measure of shape retention) of 30 GPa, approximately 4 times greater than any organic fiber. However, when the pellicle disintegrated, the Young's modulus of the sheets created from the disintegrated material was diminished by one third.

Microscopic Study of BC Papers and the Correlation with the Physical Properties

Microscopic study on micro-structures of the BC paper by using a SEM shows that the top views of the BC paper obtained from static tray and shaking flask cultures were smoother than that of the BC paper from the 5 liter stirring bioreactor (Figure 3(a-c)). The side view micrographs of the BC papers from the static tray and

shaking flask cultures show even more clearly that the fibril layered structures were organized, whereas that of the BC paper from the 5 liter stirring bioreactor was disorganized (Figure 4(a-c)). It could be said that the culture conditions affected the cellulose fibrils arrangement at microscopic level, which resulted in differences in the micro-structures morphology of the BC paper.

It was found from this experiment that oxygen transfer in the 5 liter stirring bioreactor could disturb the arrangement process of the crystalline fibril structure and also affected the physical properties of the BC paper which was indicated by it not having parchment paper characteristics, although it had the highest BC yield. The shaking flask condition, at a low shaking speed, seemed rather not to disturb the arrangement of the crystalline structure. But for the static tray condition, without disturbance, the static BC fibrils were more extended and piled above one another in a crossing manner which attributed to their strong structure, and hence to their having parchment paper characteristics.

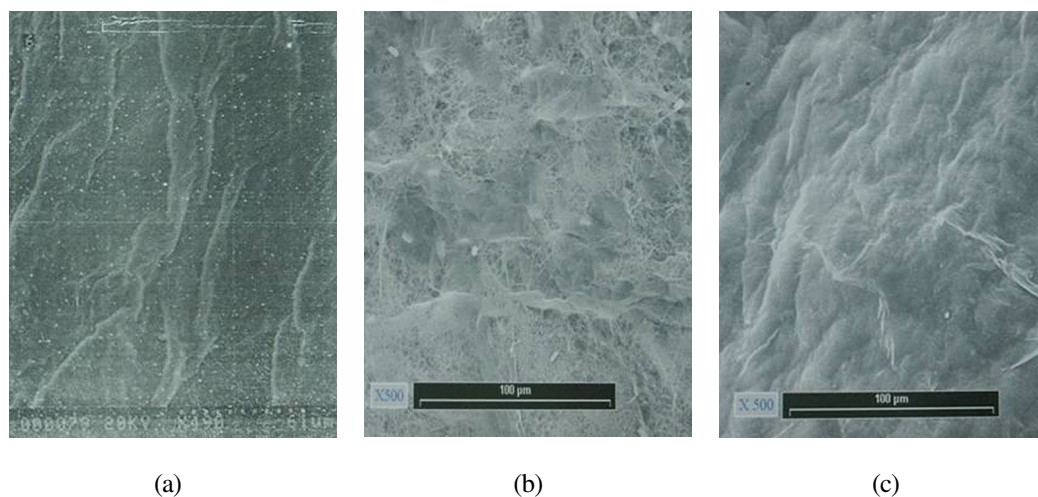


Figure 3. Scanning electron micrographs of the top views of BC paper obtained from cellulose produced by *A. xylinum* TISTR976 under three conditions; static tray (a), shaking flask (b), and 5 liter stirring bioreactor (c)

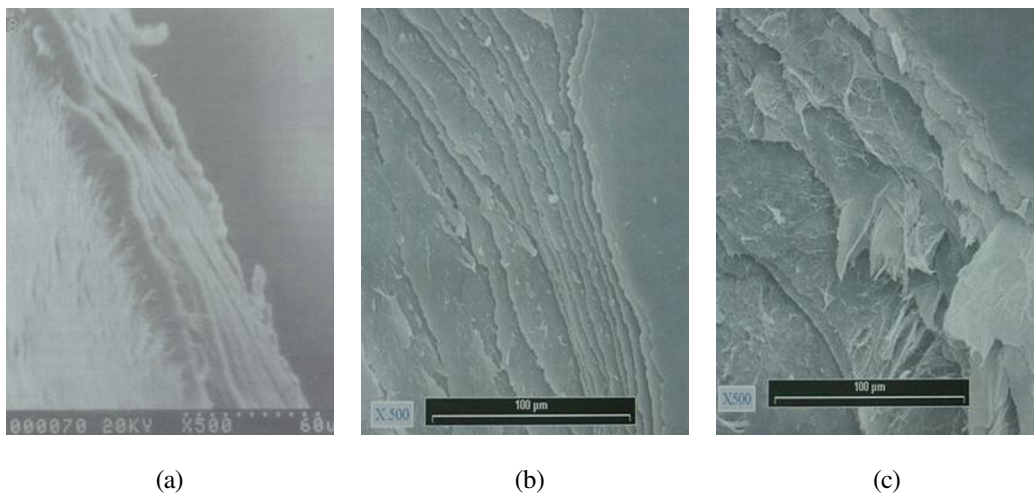


Figure 4. Scanning electron micrographs of the side views of BC paper obtained from cellulose produced by *A. xylinum* TISTR976 under three conditions; static tray (a), shaking flask (b), and 5 liter stirring bioreactor (c)

However, the lowest BC yield was obtained due to the slow growth of *A. xylinum* (data not shown).

Conclusions

A. xylinum TISTR976 was cultured in coconut juice medium to produce high yield bacterial cellulose (BC). Three culture conditions, static tray (static condition), shaking flask (shaking condition), and 5 liter stirring bioreactor (agitated condition), were applied. The results showed that culture conditions affected BC yields and the arrangement of cellulose fibrils. The BC obtained from each condition was different in macro-morphology. When the BC was processed into paper and tested for physical properties, it was found that the BC paper obtained from the static condition showed the highest wet strength retention, Castor oil penetration proof, and tearing index values. It was also found that the SEM micrographs showed the arrangement of the BC fibril layer

structure, which was correlated to the physical properties of each BC paper. This study discovered that BC paper obtained from the static condition showed a high organization of the BC fibril layer arrangement and also showed the highest physical property values as mentioned above. It could be concluded that the static condition is suitable for BC production to make BC paper due to good physical properties and having parchment paper characteristics. Besides, in an economic point of view, the static culture condition requires a low cost operation and is easy to perform; however good sanitation should be a concern because it might become contaminated during the long incubation time.

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References

- Borzani, W. and de Souza, S.J. (1995). Mechanism of the film thickness increasing during the bacterial production of cellulose on non-agitated liquid media. *Biotechnol. Lett.*, 17:1,271-1,272.
- Brown, Jr. R.M. (1992). Emerging technologies and future prospects for industrialization of microbially derived cellulose. In: *Harnessing Biotechnology for the 21st Century*. Ladisch, M.R. and Bose, A. (ed). Proceedings of the 9th International Biotechnology Symposium and Exposition. Crystal City, Virginia. American Chemical Society, Washington, D.C., p. 76-79.
- Cannon, R.E. and Anderson, S.M. (1991). Biogenesis of bacterial cellulose. *Crit. Rev. Microbiol.*, 17:435-447.
- Casey, J.P. (1960). *Pulp and Paper Chemistry and Chemical Technology*. Vol. 2, 2nd ed. Interscience, NY, 1,249p.
- Iguchi, M., Yamanaka, S., and Budhiono, A. (2000). Bacterial cellulose - a masterpiece of nature's arts. *J. Mater. Sci.*, 35:261-270.
- Jonas, R. and Farah, L.F. (1998). Production and application of microbial cellulose. *Polym. Degrad. Stab.*, 59:101-106.
- Masaoka, S., Ohe, T., and Sakota, N. (1993). Production of cellulose from glucose by *Acetobacter xylinum*. *J. Ferment. Bioeng.*, 75:18-22.
- Okuyama, A., Motoki, M., and Yamanaka, S. (1993). Bacterial cellulose IV. Application to processed foods. *Food Hydrocolloids*, 6:503-511.
- Smook, G.A. (1990). *Handbook of Pulp and Paper Terminology*. 1st ed. Angus Wilde Bellingham, Washington, D.C., 447p.
- Suwannapinunt, N., Sookson, R., and Laosangtham, D., inventors; Department of Science Service and the National Research Council of Thailand, assignee. July 24, 2001. Bacterial cellulose parchment paper with oil resistant property and process. Thai Petty Patent no. 306.
- Technical Association of Pulp and Paper Industry (TAPPI). (1998). TAPPI Press, Atlanta, Georgia. TAPPI Test method: T410 om-98, T411 om-97, T404 om-92, T456 om-96, T414 om-98, T460 om-96, T462 om-93.
- Watanabe, K. and Yamanaka, S. (1995). Effects of oxygen tension in the gaseous phase on production and physical properties of bacterial cellulose formed under static culture conditions. *Biosci. Biotechnol. Biochem.*, 59:65-68.
- Watanabe, K., Tabuchi, M., Morinaga, Y., and Yoshinaga, F. (1998). Structural features and properties of bacterial cellulose produced in agitated culture. *Cellulose*, 5:187-200.
- Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsunashi, S., Nishi, Y., and Uryu, M. (1989). The structure and mechanical properties of sheets prepared from bacterial cellulose. *J. Mater. Sci.*, 24:3,141-3,145.