The Liquid/Air Interface Area and Depth of Liquid Medium Suitable for Cellulose Production from *Acetobacter* TISTR 975

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Acetobacter xylinum TISTR 975 was cultured in a coconut water medium with added $MgSO_4$ and NH_4HPO_4. The optimum pH and amount of added sugar for the production of cellulose was found to be 4.75 and 5.1%w/v, respectively. The production of cellulose was measured on the eighth day of fermentation, while the depth of the liquid medium and surface area were varied. The depth of the medium for the highest cellulose production was 2.5 cm. The increment of medium volume by the depth of liquid medium was less effective for the production of cellulose than increasing the surface area. The highest amount of cellulose produced was 5.94 g/l from the fermentation conditions of 2.5 cm depth and liquid/air interface area of 359.12 cm^2.

Key words: Cellulose production, *Acetobacter,* liquid/air interface area.

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พื้นที่สัมผัสอากาศและความลึกของน้ำหมักที่เหมาะต่อการผลิตเซลลูโลสของ *Acetobacter* TISTR 975

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Acetobacter xylinum TISTR 975 ถูกนำมาเลี้ยงในอาหารเลี้ยงเชื้อน้ำมะพร้าวซึ่งเติม $MgSO_4$ และ NH_4HPO_4 เมื่อศึกษาหาความเป็นกรด-ด่างและปริมาณน้ำตาลที่เหมาะสม พบว่าความเป็นกรดด่างที่เหมาะสมในการผลิตให้ได้ปริมาณเซลลูโลสมากที่สุดหลังจากทำการหมักในสภาวะนิ่งเป็นเวลา 8 วัน ที่ 4.75 และปริมาณน้ำตาลที่เติมเพิ่มควรเป็นร้อยละ 5.1 เมื่อทำการหมักที่ความลึกของน้ำหมักและพื้นที่ผิวขนาดต่างๆ พบว่าความลึกของอาหารเลี้ยงเชื้อที่เหมาะสมควรเป็น 2.5 ซม และพบว่าพื้นที่ผิวมีผลต่อการสร้างเซลลูโลสของเชื้อมาก จากการเพิ่มปริมาณอาหารเลี้ยงเชื้อโดยการเพิ่มพื้นที่ผิวมีการสร้างเซลลูโลสมากกว่าการเพิ่มความลึกของอาหารเลี้ยงเชื้อ โดยปริมาณเซลลูโลสที่ผลิตได้ที่ความลึกอาหารเลี้ยงเชื้อ 2.5 ซม และมีพื้นที่ผิวหน้า 359.12 ตร. ซม. ให้เซลลูโลส 5.94 กรัมต่อลิตรอาหารเลี้ยงเชื้อ

คำสำคัญ การผลิตเซลลูโลส *Acetobacter* พื้นที่สัมผัสอากาศ

INTRODUCTION

Acetobacter xylinum is an aerobic bacterium that has ability to produce cellulose. The cellulose is produced in the form of pellicles that float at the surface of the fermenting medium during still fermentation.[1] The pellicle can be used as food, for audio diaphragms and as replacement for cellulose from plants. The pH, sugar content and alcohol content are critical for the production of cellulose.[2] During fermentation, bacterial cells get nutrients by diffusion through the pellicle.[3] Other than nutrients, *Acetobacter* needs oxygen to grow and produce cellulose. The cellulose pellicle produced by *Acetobacter xylinum* is hypothesized to be used for buoyancy for the cells so that they can receive oxygen. It had been reported that the oxygen content at the surface of the medium during the still fermentation influenced the production of the pellicle.

The aim of this study was to find the suitable surface area or liquid/air interface area and the depth of the fermentation medium for the production of cellulose.

MATERIALS AND METHODS

Acetobacter TISTR 975 was obtained from Thailand Institute of Science and Technology Research. The culture was maintained on a coconut water agar slant and stored in the refrigerator. The culture was transferred to coconut water agar slant every 14 days. It was incubated at room temperature (about 30^{o}C) for 3 days prior to keeping in the refrigerator. The active culture was prepared by transferring the stored culture into the coconut medium and incubating at 30^{o}C for 3 days prior to use as inoculums.

Preparation of medium

The soluble solid of fresh coconut water was adjusted to 3% (w/v) by addition of sucrose, boiled, then left to cool. To the coconut water, was then added $MgSO_4$ 0.05%w/v, NH_4HPO_4 0.05% (w/v) and ethyl alcohol 2% (w/v) and this was used as the basic medium. Coconut water agar was prepared by addition of 2% (w/v) agar to the basic medium.

The optimum pH and sugar concentration for cellulose production

The treatments of pH and sugar concentration of the medium were adjusted according to the Central Composite Design with 13 treatments. Acetic acid was used to adjust the pH of the basic medium to 1.2, 4.0, 5.0, 6.0 and 6.8. Sugar was added to the medium in the amounts of 2.2, 3.0, 5.0, 7.0 and 7.8% (w/v). The medium was poured into plastic container while still hot and covered with clean paper. The height of the medium and the liquid/air surface area were controlled to be the same for all fermentations. The inoculum of the culture used was 10% of the total volume of the medium. The amount of cellulose was measured at the eighth day of fermentation at room temperature.

The data were analyzed by Multiple Regression Analysis and suitable conditions for production were selected using the Response Surface Methodology.[4]

The optimum depth and surface area for the cellulose production

The amount of sugar and pH obtained from previous experiment with the same fermentation conditions were

used. The depth and surface of the liquid medium were adjusted according to the Central Composite Design with 13 treatments. The depth of the liquid medium was adjusted to 1.1, 1.5, 2.5, 3.5 and 3.9 cm. The surface of the medium was adjusted to 93.7, 114.7, 165.3, 216.0 and 237.0 cm^2. The fermentation was carried on with the same conditions as above treatments. The data were analyzed by Multiple Regression Analysis and suitables conditions for production were selected using the Response Surface Methodology.[4]

The effect of depth of the medium and surface area on the cellulose production

The optimum and conditions for the fermentation of *Acetobacter* sp. TISTR 975 were used. Three combinations of the depth and the surface area of the liquid medium were set as 7.5 cm x 165.33 cm^2, 2.5 cm x 165.33 cm^2 and 2.5 cm x 359.12 cm^2. The amount of cellulose produced was measured. The dissolved oxygen in the medium was measured by a portable oxygen meter oxi320 (WTW co., Germany). The number of cells grown was counted by TPC method using coconut water medium with 2% agar as medium. The gluconic acid was measured by a HPLC (Shimadzu LC-3A) system furnished with a Zorbox-C8 (L-3555) column, using 20 mM Phosphoric acid (pH 2.5) as solvent and a flow rate of 1 mm/min.

Determination of the cellulose content

The amount of cellulose in the pellicles was measured by boiling the harvested pellicle in 0.1N NaOH for 10 minutes. Then the pellicles were soaked in water for at least two hours and dried at a temperature of 100°C until the weight did not change. The weight of dried pellicles was expressed as cellulose weight.

RESULTS AND DISCUSSION

The optimum pH and sugar concentration for the cellulose production

The amount of cellulose produced from the fermentation of various pH and sugar concentrations were collected. The data were analyzed with Multiple Regression Analysis with $p \leq 0.05$. The relationship of cellulose (Y), pH (X_1) and sugar content (X_2) is expressed in Eq (1).

$$Y = -9.796387 + 5.087447X_1 + 1.345664X_2 - 0.478352X_1^2 - 0.074875X_2^2 - 0.125X_1X_2 \quad \ldots (1)$$

The optimum pH and sugar concentration for production of cellulose were determined by Response Surface Methodology (RSM). The three-dimension plot is shown in Figure 1. The optimum pH was 4.75 and the optimum amount of sugar added was 5.1% (w/v). The production of cellulose was 5.46 g dry cellulose/liter of liquid medium.

The composition of coconut water varies according to the location of the plantation, the maturity and the variety, but mostly contains about 2-3% of sucrose.[5] The adjustment of total soluble solid of the coconut medium was necessary. The addition of 5.1% of sucrose increased the sugar concentration in the medium to about 8.1%, and at this level of sugar, the optimum production for *Acetobacter* TISTR 975 was found. This concentration of sugar in the medium sinagreement with Alaban.[6]

The pH of the fermentation medium had an effect on the growth of *Acetobacter* sp., especially the synthesis of the enzymes and cell division.[1] The optimum pH of the *Acetobacter* TISTR 975 was found to be 4.75 which is in the range of 4-6 as reported by Masaoka et al.[7]

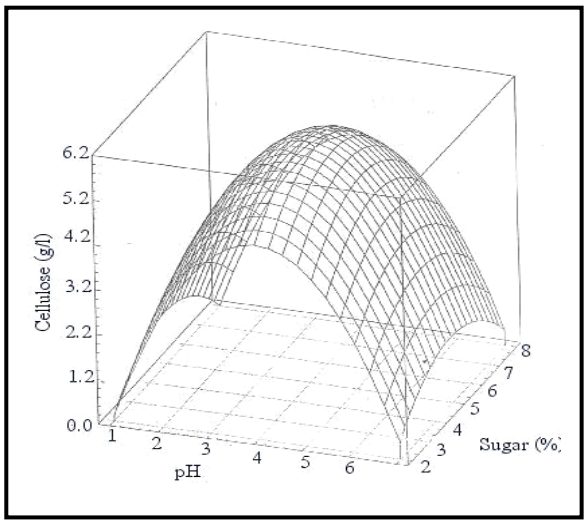

Figure 1. **Surface plot of cellulose production (g/l) at various pH and amount of sugar added (%w/v) at the eighth day fermentation of unshaken culture.**

The equation derived from this experiment can be used for the prediction of cellulose pellicle production at various conditions of pH and sugar concentration.

The optimum depth and surface area for the cellulose production

The basic medium with pH of 4.75 and 5.1% added sugar was poured into various size containers while still hot to vary the height and liquid/air interface area of fermentation medium. The cellulose pellicles were collected at the eighth day of fermentation. The data were analyzed with Multiple Regression Analysis with $p \leq 0.05$. The relationship of cellulose (Y), depth of the liquid medium (X_1) and the surface area of the medium (X_2) is expressed in Eq (2).

$$Y = - 11.731233 + 3.830596\ X_3 + 0.10496\ X_4 - 0.75879\ X_3^2 - 0.000207\ X_4^2 + 0.00296\ X_3X_4 \quad \ldots (2)$$

The optimum depth of the liquid medium and surface area for the cellulose production were determined by Response Surface Methodology (RSM). The three dimension plot is shown in Figure 2. The highest production of cellulose was found at the depth of 2.5 cm. At this depth, there was no liquid medium left in the fermentation container at the eight day of fermentation. For the surface area of the fermentation medium, the curves did not show the peak of cellulose production over the range of surface area studied.

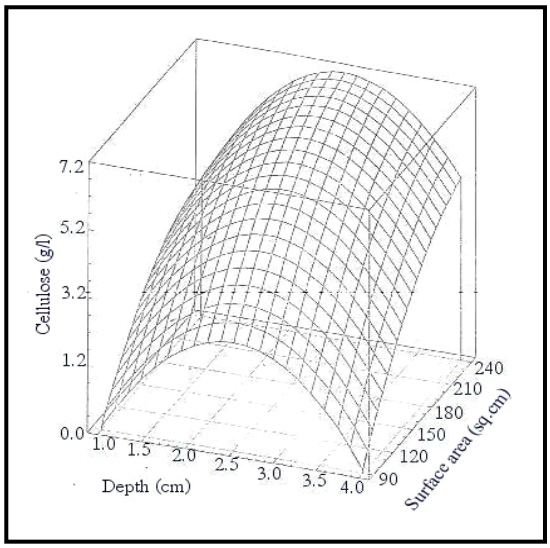

Figure 2. Surface plot on the production of cellulose (g/l) at various depth of liquid medium (cm) and surface area (cm^2) from eight day fermentation of unshaken culture.

The effect of depth of the medium and liquid/air interface area on the fermentation of cellulose production

The fermentation conditions involving liquid/air interface area, the depth of fermentation medium and cellulose production of the *Acetobacter* TISTR 975 are shown in Table 1.

Table 1. Cellulose production in various liquid medium depth and surface area of *Acetobacter* TISTR 975.

Depth (cm)	Area (cm^2)	Volume (ml)	Cellulose produced (g)	Yield (g/l)	Yield(g/cm^2)
2.5	165.33	413.3	$2.41^{a}\pm0.10$	5.83	0.015
2.5	360	900	$6.61^{b}\pm0.36$	7.34	0.018
7.5	165.33	1240	$2.87^{a}\pm0.12$	2.32	0.017

a, b Numbers in column with different superscript letters were significantly different ($P\leq0.05$)

Increasing the medium volume by increasing the liquid/air interface area affected the yield of cellulose production, while increasing the depth of the fermentation liquid medium did not affect the production yield (Table 1). These results agree with those of Masaoka et al.[7] who reported that the *A. xylinum* IFO 13693 produced the pellicle with the same cellulose content when the depth of liquid medium and surface area were varied. The thickness of the pellicles was not observed to be significantly different for all three treatments. The yield of cellulose

produced per cm^2 increased from 0.015 to 0.018 g/cm^2 as the liquid/air interface area increased from 165.33 to 360.00 cm^2. The increment of fermentation liquid depth from 2.5 to 7.5 cm also increased the yield of cellulose production to 0.017 g/cm^2. The relation of surface area and the cellulose production is shown in Figure 3 when fermentation *Acetobacter* TISTR 975 was at room temperature and the liquid medium was adjusted to a depth of 2.5 cm. It can be expressed as the equation (3) with $r^2 = 0.9852$.

$$Y = 0.0177\ X \quad \ldots (3)$$

when Y = cellulose produced (g)

X_5 = surface area (cm^2)

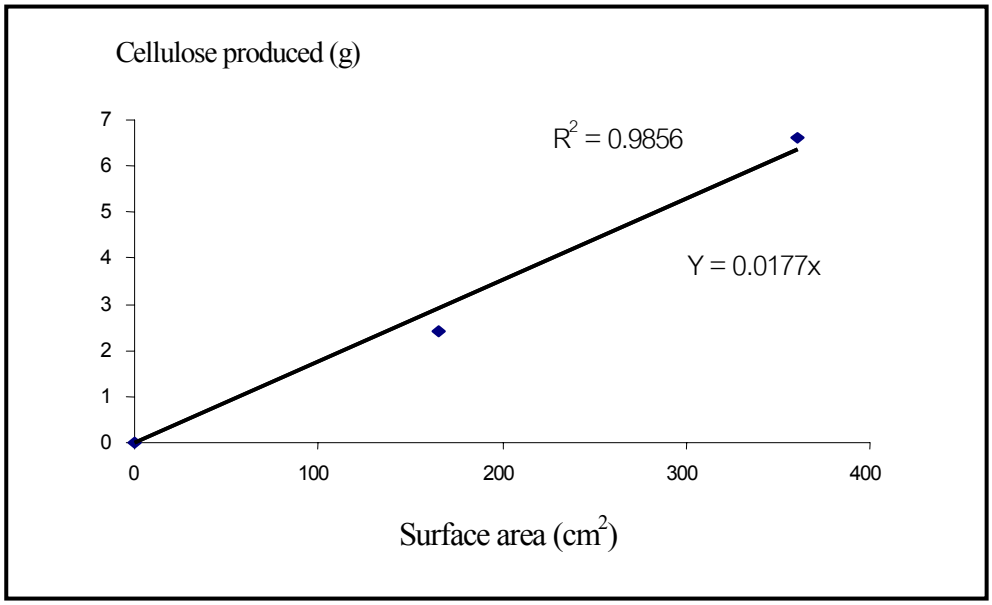

Figure 3. The effect of liquid/air interface area (surface area) and cellulose production of *Acetobacter* TISTR 975 when fermented with coconut medium at the depth of 2.5 cm under room temperature.

The dissolved oxygen in the medium at the bottom of the fermentation container with various heights and liquid/air interface areas was measured. The initial dissolved oxygen in the medium was in the range of 4.3-4.8 mg/l. The dissolved oxygen rapidly decreased to the level of 0.2 mg/l within 6 hours of fermentation. There was no difference in the oxygen depletion of all three fermentation conditions. The growth of the cultures reached a steady state after 24 hours of fermentation. The cell number in the medium was stable after 24 hours through the rest of the fermentation period, while the thickness of pellicles was growing (Figure 4).

The amount of sugar utilization in the larger liquid/air interface area was higher than in the smaller liquid/air interface area. The fermentation with a liquid/air interface area of 360.0 cm^2 and a the height of medium of 2.5 cm had a production yield of 0.661 g cellulose/g reducing sugar, while the fermentation with 165.33 cm^2 of liquid/air interface area with the same medium height had a production yield of 0.83 g cellulose/g reducing sugar. The efficiency of the production was decreased as the liquid/air

interface area increased. The reducing sugar could be utilized for the production of gluconic acid, which is the product of glucose oxidation by *Acetobacter*. There was a difference in the amount of gluconic acid produced between the three fermentation conditions. The fermentations with larger liquid/air interface area produced higher amounts of gluconic acid than the smaller liquid/air interface area (Figure 5).

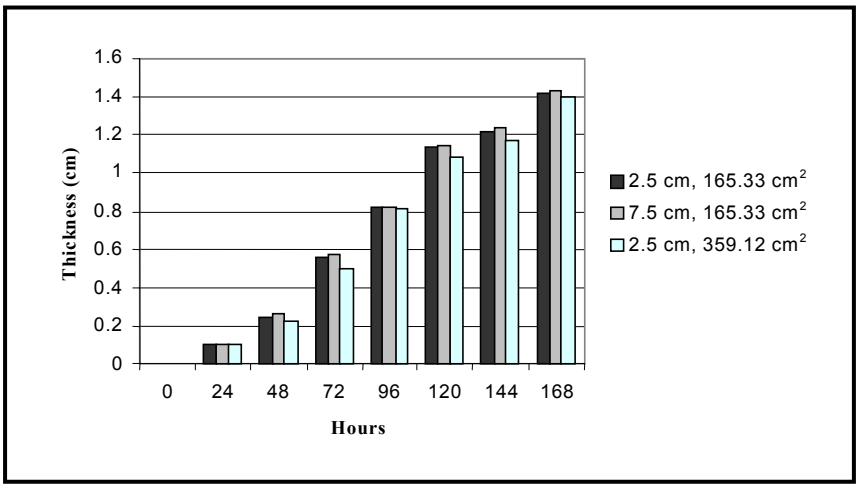

Figure 4. Cellulose production from various conditions of depth and liquid/air interface area; of 2.5 cm depth and 165.33 cm^2 of surface area, 2.5 cm depth and 360.0 cm^2 of surface area and 7.5 cm depth and 165.33 cm^2 of surface area.

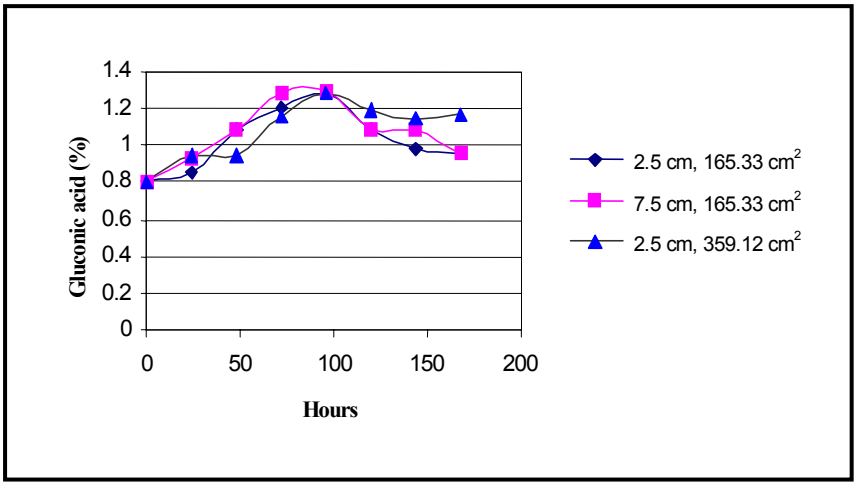

Figure 5. The amount of gluconic acid detected in the liquid medium during fermentation at various depths and liquid/air interface areas.

In addition to the liquid/air interface area and depth of the fermentation medium, the dehydration of the liquid medium was another factor. The growth of the cellulose pellicle was limited to 7-8 day fermentation because there was no liquid medium left in the container. As there was no liquid left in the system, the pellicle could start to dry up and stop the fermentation. On the other hand, more liquid medium left in the system caused a lower yield of production.

CONCLUSION

Acetobacter TISTR 975 had ability to produce cellulose pellicles in the coconut medium with optimum conditions of pH at 4.75 and sugar should be added to the coconut medium at 5.1%w/v. The increment of the medium volume by increasing the liquid/air interface area gave a higher cellulose production per unit of volume, while increasing the depth of fermentation medium did not affect the cellulose production. The larger of the liquid/air interface area also increased the amount of gluconic acid in the fermentation medium.

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REFERENCES

1. Dimaguilla, L.S. (1967) "The nata de coco I. Characterization and identity of the causal organism", *The Philippines Agriculturist.* **51(6)**, 462-473.
2. White, D.G. and Brown, R.M. Jr. (1989) "Prospect of the commercialization of the biosynthesis of microbial cellulose", In Cellulose and Wood Chemistry and Technology. Schuerch, C. ed. John Willey & Sons Inc. NY. 573-590.
3. Borzani, W and Souza, S.J. (1995) "Mechanism of the film thickness increasing during the bacterial production on non-agitated liquid media", *Biotech. Letter.* **17(11)**, 1271-1272.
4. Mason, R.L., Gunst, R.F. and Hess, J.L. (1989) "Statistical design and analysis of experiments with application to engineering and science", NY. John Wiley & Sons, Inc.
5. Grimwood, B.E. (1975) "Coconut palm products; Their processing in developing countries", Rome:Food and Agriculture Organization of the United Nations.
6. Alaban, C.A. (1962) "Studies on the optimum condition for nata de coco bacterium nata formation in coconut water", *The Philippines Agriculturist*. **45 (9)**, 450-515.
7. Masaoka, S., Ohe, T. and Sakota, N. (1993) "Production of cellulose from glucose by Acetobacter xylinum", *J. Ferment. Bioeng*. **75(1)**, 18-22.

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