Effect of Dissolved Oxygen on Cellulose Production by *Acetobacter* sp.

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The aim of this study was to examine the effect of dissolved oxygen from agitation on the production of cellulose by *Acetobacter* TISTR 975 when grown in a coconut water medium with $MgSO_4$ and NH_4HPO_4 added. The addition of sucrose at 4.98% w/v, an initial pH of 4.9 and agitation at 100 rpm were the optimum conditions for cellulose production. The cellulose production was 5.67 g/l of culture medium. Increasing rotation speed increased the dissolved oxygen in the medium and also increased the gluconic acid content. Production was reduced when excessively high oxygen content in the medium was present. The addition of carboxymethyl cellulose to the culture medium caused a decrease in oxygen content and the production of cellulose was reduced. This indicated that there was an optimum amount of oxygen that when dissolved in the culture medium would produce the highest cellulose yield. Levels of oxygen that were either below or above this amount resulted in a decrease in production.

Key words: *Acetobacter* sp., cellulose production and aerated culture fermentation.

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ผลของออกซิเจนที่ละลายต่อการผลิตเซลลูโลสโดย *Acetobacter* sp.

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งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของออกซิเจนที่ละลายต่อการสร้างเซลลูโลสของเชื้อ Acetobacter TISTR 975 ในอาหารน้ำมะพร้าวซึ่งเติม $MgSO_4$ และ NH_4HPO_4 เมื่อเลี้ยงในสภาพเขย่า จากการทดลองพบว่าภาวะที่เหมาะสมต่อการสร้างเซลลูโลส คือ อาหารน้ำมะพร้าวที่เพิ่มน้ำตาลทราย 4.98% (w/v) ความเป็นกรด 4.9 และความเร็วรอบในการเขย่า 100 รอบต่อนาที จุลินทรีย์สามารถสร้างเซลลูโลสได้ 5.67 กรัม/ลิตรของอาหารเหลว และพบว่าปริมาณออกซิเจนในอาหารเหลวมีน้อยมากหลังจากการหมักผ่านไป 72 ชั่วโมง จากการทดลองเพิ่มปริมาณออกซิเจนที่ละลายโดยการเขย่าจาก 50 เป็น 150 รอบต่อนาที พบว่าปริมาณออกซิเจนที่ละลายในน้ำหมักเพิ่มขึ้นเมื่อเขย่าเร็วขึ้น และมีปริมาณของกรดกลูโคนิคในน้ำหมักเพิ่มขึ้นแต่ปริมาณเซลลูโลสที่เชื้อสร้างกลับลดลง เมื่อทำการลดปริมาณออกซิเจนที่ละลายในอาหาร โดยการเพิ่มความหนืดของน้ำหมัก พบว่าปริมาณเซลลูโลสที่สร้างขึ้นน้อยลง ซึ่งจะเห็นได้ว่าออกซิเจนที่ละลายในอาหารเหลวในปริมาณที่เหมาะสมจึงจะให้ปริมาณ เซลลูโลสสูงที่สุด

คำสำคัญ *Acetobacter* sp. การผลิตเซลลูโลส การหมักแบบเขย่า

INTRODUCTION

Acetobacter xylinum is known as a cellulose producing bacteria. Besides the proper amount of nutrients in the medium, the culture prefers a calm and undisturbed condition to produce cellulose. This still fermentation produces cellulose at a yield that is not suitable for use on an industrial scale. A means of increasing the production of cellulose by *A. xylinum* was studied.

Acetobacter sp. is an obligate aerobe microorganism. It needs oxygen to grow and produce cellulose.[1] There are many researchers attempting to increase the cellulose production of *Acetobacter* through agitation of the medium. It was found that agitation of the culture medium did not increase the cellulose production, but rather it reduced the production yield. Also the cellulose produced in an agitated culture was formed into pellets which differ from the pellicles formed in static cultures.[2] Many researchers hypothesized that this might be because of a temporary mutation of the culture to negative cellulose producers. The increasing of these mutants in the culture would cause the reduction in the production.

The oxygen content in the medium is another factor affecting the production of cellulose. Watanabe and Yamanaka[3] found that increasing the oxygen content in *Acetobacter* culture at the liquid/air interface of the medium without agitation produced a thinner pellicle. Joris *et al*.[4] attempted to increase the cellulose production by increasing the oxygen content in the medium by shaking. However, they found that the production was reduced up to 50% and the gluconic acid content in the medium was increased.

The present study was aimed at determining the affect of increasing the amount of dissolved oxygen on the cellulose production by *Acetobacter* sp. in an agitated culture. The dissolved oxygen in the liquid medium was varied by the addition of polysaccharide in the form of carboxymethyl cellulose (CMC). As polysaccharides were added to the liquid, they increased the viscosity of the liquid and also acted as barriers to prevent oxygen from diffusing into the liquid. The addition of polysaccharides into the culture medium decreases the oxygen transfer from the air/liquid interface to the medium.

MATERIALS AND METHODS

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

Preparation of medium

The coconut medium was prepared following Punsri, *et al*.[5] (2003). Fresh coconut water was adjusted to contain soluble solids of 3% (w/v) by sucrose addition, boiled, then left to cool. The coconut water then was modified with 0.05% (w/v) $MgSO_4$, 0.05% (w/v) NH_4HPO_4 and 2% (w/v) ethyl alcohol and used as the basic medium. Coconut water agar was prepared by the addition of 2% (w/v) agar to the basic medium.

The optimum pH, sugar concentration and rotation speed for the cellulose production

The optimum pH, sugar concentration and rotation speed of the medium were examined by applying the Box-Behnken Design across 15 treatments. Acetic acid (5%) was used to adjust the pH of the basic medium to 4.0, 5.0 and 6.0. The sugar was added to the medium in the amount of 0.0, 5.0 and 10.0% (w/v). The medium was poured into 250 ml, sterile Erlenmeyer flasks. The inoculum of the culture was 10% of the total volume of the medium. The cultures were placed on a rotary shaker set at 50, 125 or 200 rpm for 8 days at room temperature. The amount of cellulose was measured after the eighth day.

The data were analyzed by Multiple Regression Analysis and the most suitable condition for production was selected using Response Surface Methodology.[6]

The effect of dissolved oxygen on the cellulose production

The optimum conditions for the fermentation of *Acetobacter* sp. TISTR 975 were used. The shaking speeds used were 50, 100 and

150 rpm. 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 the TPC method using coconut water with 2% agar as medium. The gluconic acid was measured by an HPLC (Shimadzu LC-3A) system furnished with Zorbox-C8 (L-3555) column, using 20 mM Phosphoric acid (pH 2.5) as solvent and a flow rate of 1 ml/min.

CMC 0.3, 0.2 and 0.1% w/v was introduced to the culture medium. The cultures were shaken for 6 days on a rotary shaker at the determined optimal speed for cellulose production. The cellulose production, amount of dissolved oxygen, viable cells, amount of reducing sugar and viscosity of the medium were determined.

Determination of the cellulose content

The amount of cellulose in the pellicles was measured by boiling the harvested pellicle in 0.1 N NaOH for 10 minutes. The pellicles were then soaked in water for at least two hours. Then, they were dried at a temperature of 100°C until the weight no longer changed. The weight of the dried pellicles was expressed as cellulose weight.

RESULTS AND DISCUSSION

The optimum pH, sugar concentration and rotation speed for the cellulose production

The amount of cellulose produced from the fermentation of various pH and sugar concentrations was determined. The data were analyzed with Multiple Regression Analysis with $p \leq 0.05$. The relationship of the cellulose produced (g/l) (Y) and pH (x_1), the amount of added sugar (%) (x_2) and rotation speed (x_3) is expressed in equation. 1 with $r^2 = 0.8932$.

$$Y = -14.770287 + 7.832375x_1 + 0.190175x_2 + 0.028506\,x_2 - 0.805583x_1^2 - 0.0518x_1x_2 - 0.002543x_1x_3 - 0.0190715x_2^2 + 0.000593x_2x_3 - 0.000136x_3^2 \quad \text{... (1)}$$

The statistical analysis of the data showed that only the independent variables of pH (x_1) and rotation speed (x_3) were significant ($p \leq 0.05$). To find the optimal conditions for cellulose production, partial differentiation by differentiation of value Y against x_1, x_2 and x_3 was performed assuming that dY/dx_1, dY/dx_2 and dY/dx_3 were equal to 0. We got the amount of added sugar (x_2) = 4.98%. By placing the value of x_2 in equation 1, we got the prediction equation as equation 2.

$$Y = -14.770287 + 7.832375x_1 + 0.028506x_3 - 0.805583x_1^2 - 0.000136x_3^2 \quad \text{... (2)}$$

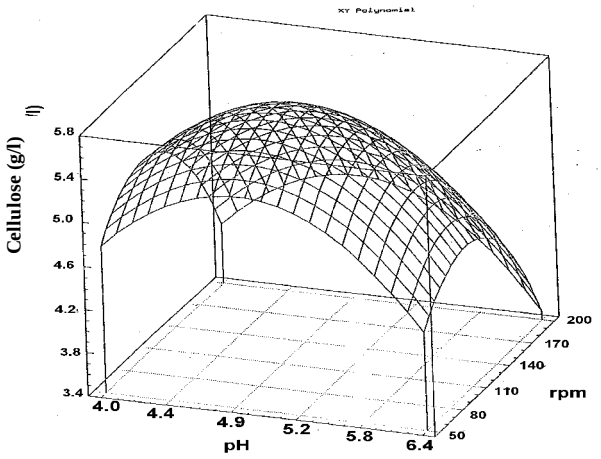


Figure 1. Surface plot of cellulose production (g/l) at various pH and amount of sugar added (%w/v) at the eighth day of fermentation of shaken or agitated culture.

The surface plot of equation 2 showed the optimum pH was at 4.9 and optimum rotation speed was 100 rpm (Figure 1). The production of cellulose was 5.67 g of dry cellulose per liter of liquid medium when the amount of sugar added to the medium was 4.98% (w/v).

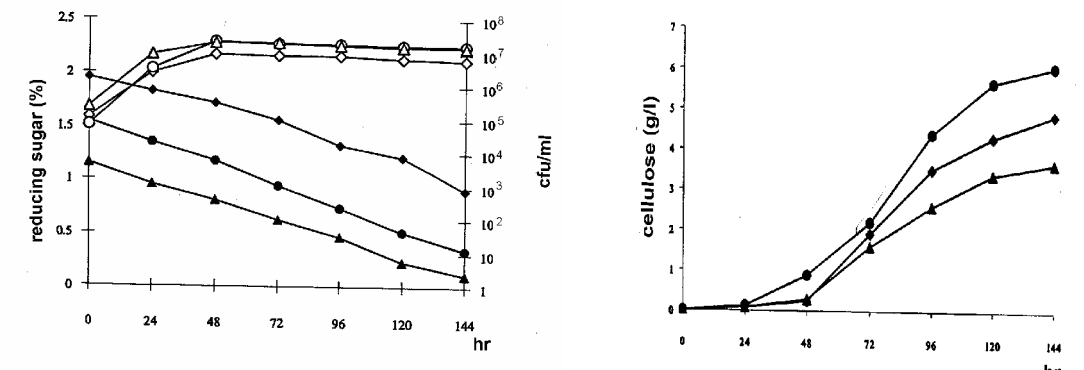
The composition of the coconut water varied depending on the location of the plantation and the maturity and variety of the coconut but most contained 2-3% sucrose.[7] An adjustment of total soluble solids of the coconut medium was necessary. The coconut water in this experiment was adjusted to 3% total soluble solids. The addition of 4.9% sucrose increased the sugar concentration in the medium to about 8%, the optimum level for cellulose production by *Acetobacter* TISTR 975. This concentration of sugar was agreed with Alaban.[8]

The pH of the fermentation medium had an effect on the growth of *Acetobacter* sp. Especially effected were the synthesis of the enzymes and cell division.[9] The optimum pH of the *Acetobacter* TISTR 975 was found to be 4.98 which was in the range of 4-6 for *A. xylinum* as reported by Masaoka *et al*..[10]

The effect of dissolved oxygen on the cellulose production

The dissolved oxygen in the medium was varied by increasing the speed of shaking. It was found that 100 rpm gave the highest amount of cellulose, 5.67 g/l of coconut water medium (Figure 2). This optimal agitation speed agreed with the data as predicted above, but the amount of cellulose predicted by the equation at 100 rpm was only 4.02 g/l.

It was found that solutions processed at 50 rpm showed the smallest number of cells while the other samples had the same higher number of cells. This could result from dissolved oxygen increasing the growth of the cells. After cell number optimization, excess oxygen in the medium did not further increase cell production. The amount of reducing sugar utilized by the culture in all conditions was the same. The reducing sugar in the medium of all conditions was reduced from the initial amount to about 1%.



(a) **(b)**

Figure 2. The reducing sugar content, cell number in the coconut medium of *Acetobacter* TISTR 975 (a), the amount of cellulose (b) produced at various rotation speeds; where reducing sugar and cell number are shown as —◆—, —◇— at 50 rpm; —●—, —○— at 100 rpm; and —▲—, —△— at 150 rpm, respectively.

The amount of oxygen dissolved in the medium of the 150 rpm culture was the highest, while there were only small amounts left in the 50 and 100 rpm cultures (Figure 3). Increasing the agitation speed of the culture increased the amount of gluconic acid content in the culture medium (Figure 4). In the 150 rpm culture, the excess oxygen dissolved in the medium increased the accumulation of gluconic acid and the cellulose production was reduced.[11] The sample treated at 50 rpm showed undetectable levels of oxygen dissolved in the medium after 96 hrs of fermentation and found low amounts of gluconic acid. Also cellulose production was the lowest; this might be because there was an inadequate amount of oxygen for cell activities and cellulose production.

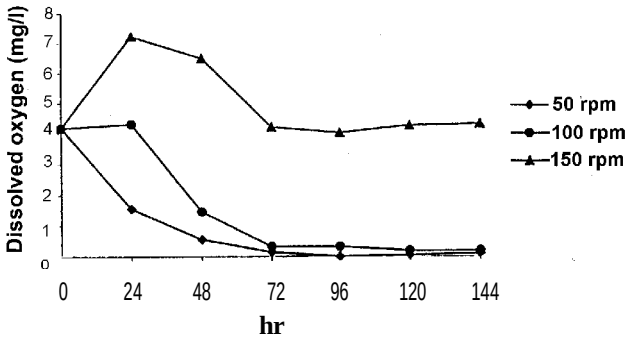


Figure 3. Dissolved oxygen in the coconut medium when *Acetobacter* TISTR 975 was cultured under agitation.

Even though, *Acetobacter* sp. is an obligate aerobe bacterium,[1] it needs oxygen to grow. The amount of oxygen in the medium that exceeded that needed by the culture might function as a proton acceptor. It might oxidize glucose to gluconic acid instead of being utilized for cellulose production as shown in the metabolic pathway presented by Ross, *et al.*[12] As the glucose was converted to substances which were not associated to the cellulose production pathway, this caused the reduction of cellulose production.

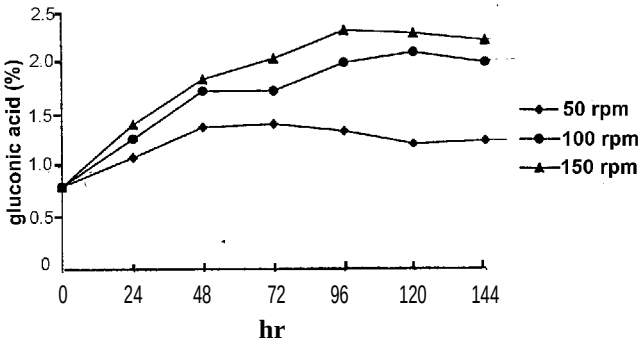


Figure 4. The gluconic acid content in the coconut medium when *Acetobacter* TISTR 975 was cultured at various rotation speeds.

The addition of CMC of 0.1, 0.2 and 0.3% to the coconut water medium increased the viscosity of the medium to 1.58, 1.82 and 2.06 mPa.s, respectively. Increasing of the medium viscosity decreased the diffusion of oxygen into the culture medium. As the cultures were shaken at the same rotation speed, the cultures with added CMC were running out of oxygen faster than the ones without CMC added. The cell numbers of the control (without CMC added) medium was 4 times higher than that of the medium with CMC added. The utilization of reducing sugar by the cultures was also found to be lower than in the medium without CMC added (Figure 5). The cultures with the addition of CMC showed decreasing cellulose production (Figure 6).

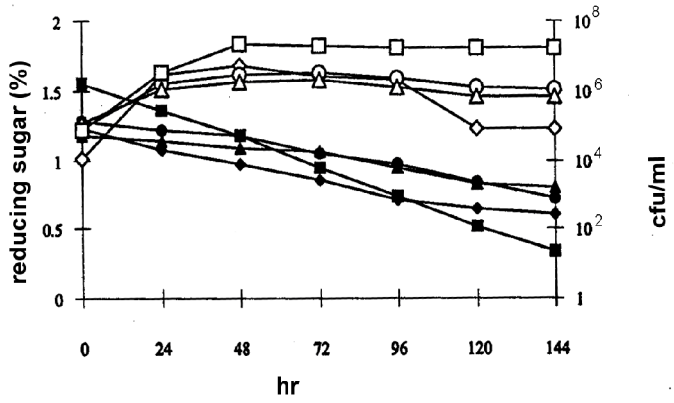


Figure 5. The reducing sugar content, cell number in the coconut medium of *Acetobacter* TISTR 975 produced when shaking at 100 rpm; where reducing sugar and cell number are depicted as ◆, ◇ at 0.1%CMC; ●, ○ at 0.2%CMC; ▲, △ at 0.3% CMC; and ■, □ control, respectively.

The medium with CMC added at 0.1 and 0.2% showed higher cellulose production than control in the first 72 hrs of fermentation. This might be the effect of CMC. Ben-Hayim and Ohad[13] reported that CMC might induce the polymerization of glucose in *A. xylinum* by up to 30%. As the fermentation continued, the cellulose production in those conditions was reduced and the detected oxygen content in the medium was very low after 72 hrs of fermentation (Figure 6).

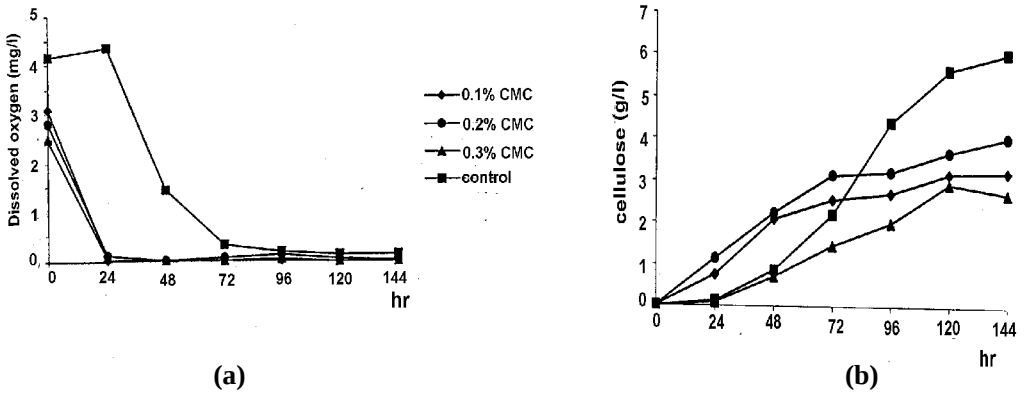


Figure 6. The dissolved oxygen (a) and cellulose production (b) in the cultured medium of *Acetobacter* TISTR 975 when the amount of carboxymethyl cellulose was varied.

This could also indicate that the amount of dissolved oxygen in the medium affected the cellulose production. The *Acetobacter* culture needs oxygen at different concentrations to produce energy for growing and to produce cellulose. At the initial state of fermentation, the dissolved oxygen in the medium with added CMC was limited. The culture might produce the cellulose to float the cells to the surface of the medium. Cellulose production in the medium with 0.1% and 0.2% CMC added was higher than the control (without CMC) sample in which dissolved oxygen was detectable. After 48 hrs of fermentation, the dissolved oxygen was found to be very low. An increase in cell numbers was not found while the utilization of reducing sugar by the culture was less than that of the samples without CMC added. In the control culture, the shaking of the culture provided oxygen for increasing the number of cells at the initial state of the fermentation. After 72 hrs of fermentation, when small amounts of

dissolved oxygen were detected, the culture produced cellulose instead of increasing the amount of cells.

CONCLUSIONS

The dissolved oxygen in the culture medium was a factor affecting cellulose production. The *Acetobacter* TISTR 975 needed a proper amount of dissolved oxygen in the medium to maximize cellulose production. Too much dissolved oxygen content in the medium increased gluconic acid content in the medium and reduced cellulose production. Too low an amount of dissolved oxygen content in the medium could not provide enough oxygen for the culture to grow and that caused the reduction of cellulose production.

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