Study of *Acetobacter xylinum* FNCC 0001 fermentation kinetics using artificial media containing various carbon and nitrogen concentrations

A M Sari, F A Budianto, A Nursiwi, A P Sanjaya, R Utami, M Z Zaman
Department of Food Science and Technology, Universitas Sebelas Maret, Ir. Sutami Street 36A, Surakarta 57126, Indonesia

Email: ardhea_ms@staff.uns.ac.id

Abstract. The starter medium’s proper composition was needed to obtain the best bacterial growth medium. Therefore, coconut water artificial medium was used with 3 variations of glucose and peptone concentrations as carbon and nitrogen sources respectively to obtain the best formulation in *Acetobacter xylinum* FNCC 0001 fermentation kinetics. Both concentrations’ effects were evaluated using the fermentation kinetics parameter. The growth curve, bacterial cellulose (BC) yield, pH change, and fermentation kinetics were observed at 24 points within 7 days. Furthermore, the variations were 6.612 g/L glucose and 16 g/L peptone (F1), 9.541 g/L glucose and 21 g/L peptone (F2), and 17.172 g/L glucose and 26 g/L peptone (F3). The results showed that carbon and nitrogen concentrations’ variations affected the bacterial growth and fermentation kinetics. The F1 treatment gave the best fermentation kinetics based on the best cell growth kinetics parameters including specific growth rate (Rx or µ), doubling time (Td), multiplication degree (n), and maximum specific growth rate (µ max).

Keywords: bacterial cellulose, C/N ratio, coconut water medium, and growth rate.

1. Introduction

Cellulose is a natural biopolymer widely used in production sectors like food and pharmaceutical industries. It can be extracted from plant or synthesized by various bacteria species [1]. Studies have reported cellulose produced by various bacteria species such as in the genera *Gluconacetobacter* (formerly *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, *Salmonella*, *Enterobacter* and *Escherichia* [2]. Among all mentioned, *Acetobacter xylinum* is the most studied and effective to produce bacterial cellulose (BC) in both artificial and non-artificial medium through oxidative fermentation using various carbon sources viz. sucrose, fructose, glucose, invert sugar, glycerol and ethanol[3]. This is a gram-negative and acetic acid bacterium that grows at pH 3.5 and produces BC at pH 4.0-5.0[2].

In South-East Asia countries such as Philippine and Indonesia, BC is used to make *Nata de Coco*, a kind of dessert produced by *Acetobacter xylinum* fermentation on coconut water medium. Recently, *nata de coco* is largely manufactured at many small industries in Indonesia and exported as a healthy food[2] [4]. However, some problems encountered are related to this food’s productivity, therefore various studies focused on increasing the yield by screening high-producing BC strains, developing Low-cost media, optimizing cultivation and downstream process [5]. Some of these small industries at Central Java, Indonesia also had the same problem especially low production quantity due to the lack of
bacterial growth on the starter medium. Hence, starter and fermentation media composition plays important role for cell proliferation and metabolites biosynthesis.

As a major component in culture medium, carbon and nitrogen sources have significant effect on cell proliferation and metabolite biosynthesis. Previous study reported peptone as the best nitrogen source for BC production, and glucose as favorable carbon source. In addition, the carbon to nitrogen (C/N) ratio of 5.39 has been stated to promote bacteria proliferation while 6.31 encouraged BC synthesis, therefore making it a crucial parameter [5]. CN-ratio’s effect on cell growth and metabolite production have been evaluated on Acetobacter xylinum NUST4.2 liquid culture [5]. But, there has been no report about substrate concentration’s effect on fermentation kinetic parameters in coconut water artificial media used for nata de coco’s production in Indonesia. The mature coconut water contains many minerals required for Acetobacter xylinum growth such as sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and phosphorus (P) [6]. This study aimed to observe the fermentation kinetic of A. xylinum FNCC 0001 using coconut water artificial media with the addition of various glucose and peptone concentrations. A proper carbon and nitrogen concentrations in culture medium may be applied to solve the small industries’ low productivity problem in Central Java.

2. Experimental
2.1. Preparation of microorganism and artificial medium
2.1.1. Microorganism and inoculation. Acetobacter xylinum FNCC 0001 was used in agar slant obtained from Food and Nutrition Centre of Excellent, Gadjah Mada University. The stock culture was grown in Hestrin-Schramm (HS) medium containing 2.0% D-glucose, 0.5% peptone, 0.5% yeast extract, 0.27% Na₂HPO₄ anhydrate and 0.115 % (w/v) citric acid in distilled water with pH adjusted to 6.0. This was inoculated in 250 ml conical flask containing 50 ml HS medium and incubated for 48 h under static conditions [7]. Then the seed culture was inoculated in coconut water artificial media with addition of various glucose and peptone concentrations.

2.1.2. Preparation of artificial media. Coconut water with an identified total carbon and nitrogen was added to glucose and peptone to attain 5.39 C/N ratio [5]. There were 3 artificial media formulations with different glucose and peptone concentrations namely 1 L of 4.1° Brix coconut water, 6.612 g/L glucose and 16.0 g/L peptone (F1), 1 L of 4.5° Brix coconut water, 9.541 g/L glucose and 21.0 g/L peptone (F2), and 1 L of 4.0° Brix, 17.172 g/L glucose and 26.0 g/L peptone (F3).

2.2. Fermentation in artificial media.
Each artificial medium’s pH was adjusted to 5 by adding 98% acetic acid. The fermentation was conducted in 250 ml flasks filled with 30 ml of artificial media, followed by sterilization and inoculation with 5% (v/v) seed culture prepared in HS medium containing 10⁷ cells/ml of Acetobacter xylinum. This lasted for 168 hours (room temperature) and was sampled for analysis every 2 hours during the first 12 hours, then every 3 hours at the 12th-24th hour, every 8 hours at the 24th-48th hour and finally at every 12 hours during the 48th -168th hour, but the whole fermentation was carried out in 3 batches.

2.3. Analytical method
2.3.1. The growth curves. A. xylinum’s cell number was analysed to observe the growth curve by measuring the fermentation media’s absorbance at 660 nm [7]. Then, the data were compared to the Total Plate Count (TPC) in HS-agar medium using spread plate method with incubation at 30°C for 4 days [8]. Gravimetric method was used to make a standard biomass curve in g/L dried cell weight (DCW) and conducted by centrifugation of 7 ml medium in 15 ml centrifuge tube at 8000 rpm for 10 min [9]. The pellet was dried at 105°C for 10-12 h and weighed [10], while the sample’s DCW was obtained by measuring its medium’s absorbance at 660 nm, then compared to the TPC and gravimetric method.
2.3.2. Analysis of substrate residue. Reducing sugar or substrate residue was analysed by spectrophotometry method using 3,5-dinitrosalicylic acid (DNS). Furthermore, 24 samples were also used in this process during 7 days fermentation [11].

2.3.3. Analysis of Yield. Bacterial cellulose (BC) produced in each fermentation bottle was analyzed by filtration and purification. The agglomerate in the bottles was filtered and purified by boiling in 0.1N NaOH at 80°C for 20 minutes, then washed with water [12]. The yield’s data were obtained by weighing purified BC from the previous step after being dried overnight at 105°C[13].

3. Result
3.1. The Growth Curve of Acetobacter xylinum FNCC 0001. Glucose and peptone composition’s variation in the medium affected Acetobacter xylinum FNCC 0001 growth. It can be seen from the lag, exponential and stationary phases during 168 h fermentation. The F2 formula containing 9.541 g/L glucose and 21 g/L peptone showed the shortest lag phase (2 h) but had the longest exponential from 2nd h until 24th h then followed by stationary at 24th hour. Conversely, F3 showed the longest lag phase (8h), followed by exponential until the 18th hour and stationary from this time until the fermentation ended. Meanwhile, the F1 treatment containing 6.612 g/L glucose and 16 g/L peptone showed the lag phase until 6 hours, followed by exponential from the 6th to 18th hour and stationary from the 18th hour until the fermentation ended.

![Figure 1. The Growth Curve of Acetobacter xylinum FNCC 0001 in various media formulations F1 (o), F2 (△), and F3 (□)](image)

The F3 has a long lag phase possibly due to glucose and peptone addition with the highest concentration affecting cell culture adaptation’s length at the beginning of fermentation. When compared to the one in HS-medium composition (starter growth media), the total carbon in F3 was much higher to ensure Acetobacter xylinum cells required a longer adjustment time than the other treatments.

Overall, the three treatments’ growth curve graph shows an increase in the total dry cell biomass which is directly proportional to increasing glucose and peptone concentrations’ addition. F3 had the highest dry cell biomass weight which was 1.575g/L medium (132nd hour), followed by F2 with 1.492 g/L medium (132nd hours) and F1 with 1.440 g/L medium (84th hour). The Acetobacter xylinum’s expected starter growth medium formulation reached the stationary phase in the shortest time. Based on the results, F1 reached the stationary phase fastest than the others.

3.2. Yield. Bacterial cellulose (BC) is a secondary metabolite produced by Acetobacter xylinum with glucose precursors formed by gluconic acid release [14]. The yields on artificial coconut water media with glucose and peptone concentrations’ variations as carbon and nitrogen sources respectively during
fermentation can be seen in Figure 2. The three variations indicated that the yield started forming after the 24th hour (stationary phase) and its purification analysis was carried out at 32nd hour. According to the growth curve in Figure 1, bacterial cells begin to form BC at the end of the exponential phase [14].

![Figure 2. The Yield of Bacterial Cellulose (BC). Media formulations F1 (□), F2 (△), and F3 (x) ](image)

BC production was greater in the F1 treatment than F2 until the 108th hour, but at the end of fermentation F1 had lower production of 1.9722 g/L compared to 3.0533 g/L in F2. This was because according to the cell growth curve (Figure 1), F1 reached the end of stationary phase faster than F2 to ensure *Acetobacter xylinum* produced more BC and obtained a higher yield. The yield in the three treatments is directly proportional to the increase in carbon and nitrogen sources’ concentrations because glucose is the main component in BC formation. This is supported by Zhang *et al.* (2016) which stated that glucose is the main precursor used for bacterial cellulose preparation[15].

### 3.3. pH Analysis

*Acetobacter xylinum* is an aerobic acetic acid bacterium growing well at pH 3.5-7.0[16]. Because the optimum pH for BC production and cell growth was 5.0[17], the artificial coconut water media’s pH was adjust to 5.0. At the early fermentation (6th h) the pH of all media formulations was stable and decreased significantly afterwards until 60th h. Then, it slightly increased until fermentation ended (Figure 3).

![Figure 3. Changes in the Media’s pH during Fermentation. Media Formulations F1 (o), F2 (△), and F3 (□).](image)
Decrease in pH indicated the cells were growing fast and producing gluconic acid from sugar metabolism[17]. After glucose concentration in the media tends to diminish, Acetobacter xylinum used gluconic acid to produce BC and caused slight pH increase at the end of exponential phase.

3.4. Kinetics Fermentation. Acetobacter xylinum’s fermentation kinetics parameter in artificial coconut water with glucose and peptone concentrations’ variations can be seen in Table 1. The highest cell-specific growth rate was shown as 0.0900 g/L/hour in F1 treatment, followed by 0.0877 g/L/hour in F3 and the lowest was 0.0549 g/L/hour in F2. The difference in the growth rate in the three treatments varied due to the growth curve pattern and length of each treatment’s exponential phase.

The doubling time (Td) is the time needed by a microbial biomass to duplicate or fold the initial population. F1 gave a Td value of 7.737 hours, while F2 and F3 gave 12.662 and 7.904 hours respectively. The longer doubling time in F2 showed that Acetobacter xylinum cell growth was slower compared to other treatments.

The multiplication degree (n) is the quotient between X/Xo which shows the number of cell multiplications that occur during fermentation process [18]. The F1 gave the lowest n value of 1.550 times, while F2 had 1.742 and F3 had the highest which was 2.023.

Substrate degradation rate (Rs) of F1 gave the greatest value (0.0503 g/L / hour), while the F2 gave the lowest (0.0303 g/L / hour) and F3 gave 0.0436 g/L / hour. In terms of product (BC) formation rate (Rp) F1 gave the lowest value of 0.0122 gram / liter / hour, while F2 gave 0.0303, and F3 gave 0.0365.

Cell formation ratio (Yx / sYx/s) is the ratio of cell biomass formation to glucose substrate’s amount. Substrate usage ratio (Yp / s) is the ratio of BC yield formation to glucose substrate utilization determined by (P-Po) / (So-S) method. Meanwhile, the product formation ratio (Yp / x) is the BC yield ratio to the cell biomass determined by (P-Po) / (Xo-X). The yield ratio coefficient is used to measure the efficiency of substrate conversion into cell biomass and the desired product [17]

| Kinetic Parameters                      | Formulation | Unit                  |
|----------------------------------------|-------------|-----------------------|
| R or μ (specific growth rate)          | F1          | F2                    | F3                    |
| Td (doubling time)                     | 7.737       | 12.623                | 7.904                |
| N (degree of multiplication)           | 1.550       | 1.742                 | 2.023                |
| Rs (substrate utilization rate)        | 0.0503      | 0.0303                | 0.0436               |
| Rp (product formation rate)            | 0.0122      | 0.0303                | 0.0365               |
| Y_{s/k} (ratio of cell to substrate)   | 1.522       | 1.518                 | 1.613                |
| Y_{p/s} (ratio of product to substrate)| 0.309       | 0.456                 | 0.385                |
| Y_{p/k} (ratio product to cell)        | 2.018       | 2.865                 | 4.192                |
| μ max (maximum specific growth rate)   | 0.0909      | 0.0597                | 0.0898               |
| σ (specific product formation rate)    | 0.0113      | 0.0211                | 0.0223               |

Cell formation ratio’s value in F1 treatment was obtained at 1.522 grams of biomass / gram of glucose, but F2 gave 1.518 and F3 gave 1.613 as the highest value. The lowest substrate usage ratio was found in F1 as 0.309 grams BC / gram glucose, while in F2 was 0.456 and F3 was 0.385. Based on the product formation ratio, F1 showed the lowest efficiency of 2.018 grams BC / gram cell biomass, F2 showed 2.865 and F3 showed 4.192.

The maximum specific growth rate (μ max) determined the highest cell growth rate of Acetobacter xylinum during the fermentation process. Moreover, F1 had the highest μ max (0.0909 / hour) when compared to the other treatments. The F2 had the lowest (0.0597 / hour) and F3 had 0.0898 / hour. Regarding specific formation rate (σ) of BC, F1 gave the lowest value as 0.0113 / hour, while F2 gave 0.0211 / hour and F3 gave the best as 0.0223 / hour.
4. Conclusion
The variation in concentrations of added glucose and peptone as carbon and nitrogen sources respectively in artificial coconut water media affected *Acetobacter xylinum* FNCC 0001 growth and kinetic parameters. With reference to kinetic parameters, the F1 formulation was the best for the bacterial growth.

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