Kinetic study of *Escherichia coli* BPPTCC-EgRK2 to produce recombinant cellulase for ethanol production from oil palm empty fruit bunch

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Abstract. Oil Palm Empty Fruit Bunch (OPEFB) is an abundant biomass resource in Indonesia, which contains 46.77% (w/w) of cellulose. The high cellulose content of OPEFB can be used as a substrate for bacteria cultivation to produce cellulase. By using OPEFB as an alternative substrate, the production cost of cellulase in industrial scale can be suppressed. However, currently there are no available research that simulate a cellulase production plant design. Prior to simulating the cellulase plant design, kinetic studies of bacteria used in cultivation are needed to create an accurate simulation. In this research, kinetic studies of *E. coli* BPPTCC-EgRK2 growth were examined with the Monod approach to get the Monod constant (Ks) and maximum specific growth rate (μmax). This study found that *E. coli* BPPTCC-EgRK2 have μmax and Ks of 1.581 and 0.0709 respectively. BPPTCC-EgRK2 produce intracellular cellulase, thus gave linear correlation between cell concentration and cellulase production.

1. Introduction

As a tropical country, Indonesia is rich with potential biomass that can be utilized as bioenergy feedstock. Bioenergy can be used as fuel in the form of liquid (biodiesel, bioethanol) or gas (biogas). Indonesia Ministry of Energy and Mineral Resources stated that Indonesia's bioenergy potential derived from biomass waste is estimated to reach 49,810 MW. However, bioenergy utilization until today has only reached 1,618 MW or about only 3.25% of the total potential. One form of alternative energy that is environmentally friendly as well as promising in the future is bioethanol. The demand for bioethanol in the world continues to increase from year to year, where the rate of global bioethanol production from 13.2 billion gallons in 2007 is increased to 25.7 billion gallons in 2015 [1].

To produce bioethanol from biomass, there are some pre-treatment that has to be done on the biomass before converting it to bioethanol with fermentation. One of the hydrolysis agents to pre-treat the lignocellulosic biomass is cellulase enzyme. As the bioethanol needs increases, the needs for cellulase will also increase. This can be seen from the market revenue of the world biofuel enzymes (cellulase and amylase), which keeps increasing from 2010 to projection year 2018 [2]. Not only the global level, but also domestic cellulase demand are increasing. Enzyme consumption for local industries is
estimated to reach around 2,500 tons in 2015 with an import value of Rp187.5 billion [3]. However, according to Juniarto as quoted by Agency for Assessment and Application of Technology in 2013, 99% of the enzymes used for industries in Indonesia are imported from other countries. The import value of the cellulose enzyme is estimated to reach 121.85 billion rupiah and will continue to increase with a rate of increase of 6.67% per annum. This is unfortunate, as there is enormous potential in Indonesia’s natural resources that should be able to meet those needs.

One example of Indonesia’s natural resource potential is oil palm. The processing of palm leads to the formation of several by-products and residues that have economic potential. Empty fruit bunches (EFBs) are solid residue and they have a high cellulose content, at 46.77% of EFB dry weight [4]. In this case, the high cellulose content of EFB is used as a substrate for bacteria cultivation to produce cellulase. By using EFB as an alternative substrate, the production cost of cellulase on an industrial scale can be suppressed.

Currently, most cellulase enzymes are produced from saprophytic microorganisms [5]. However, industrial applications often require a cost-effective production of enzymes on a large scale, which is a common bottleneck. In producing cellulase enzymes, bacteria have several advantages over fungi, which are faster in growth rates and easily genetically engineered, although the number of enzymes produced is not as much as mushrooms [5]. In this study, bacteria were used to produce cellulase from the genetically engineered *Escherichia coli* BPPT-CC EgRK2 isolates.

Prior to simulating the cellulase production in large scale, kinetic studies of bacteria used in cultivation are needed to create an accurate simulation. In this research, kinetic studies of *E. coli* BPPTCC-EgRK2 growth are examined with the Monod approach to get the Monod constant (Ks) and maximum specific growth rate (μm). After determining the growth kinetics parameter of *E. coli* BPPTCC-EgRK2, one can design the shape and size of the fermentor to produce cellulase enzyme by *Escherichia coli* BPPT-CC EgRK2 on an industrial scale.

2. Materials and Methods

This research is divided into 3 stages: the preparation stage, the making of growth curve, the last is the stages are determining kinetic parameters and simulation. The preparatory stage includes a literature study of theory as well as previous research related to the study of *Escherichia coli* growth kinetics. After preparation of literature, tools and materials finished, medium of Luria Bertani (LB) for fermentation is prepared and *Escherichia coli* BPPT-CC EgRK2 is rejuvenated. At the stage of making growth curves, *Escherichia coli* BPPT-CC EgRK2 is cultured at optimum conditions with glucose substrate. Glucose substrate is varied with 1 g.L⁻¹ and 10 g.L⁻¹ concentration. While the fermentation process is going, the samples is analysed to get the optical density, dry cell weight, and glucose concentration data. In the final stage, microbial growth analysis is done through Monod approach and data validation is performed. In addition, a review of batch production method and fed batch is done by using SuperPro Designer 9.0 application.

The culture was obtained from BPPT (Indonesia’s Agency for Assessment and Application of Technology), Serpong, Indonesia. Growth and culture conditions were referred to the previous research [6]. 1-2 loopful of *E. coli* BPPTCC Eg-RK2 was inoculated into a 100 ml of LB Erlenmeyer flask containing 25 mL of Luria Bertani medium made from (g.L⁻¹): yeast extract, 5; peptone, 10; and NaCl, 10. The pH of each medium was adjusted to 7.0 with 0.1N NaOH. Sterilization was done for 15 min at 121 °C, 1.2 atm. This seed culture was grown for 6 hours at 37°C with agitation at 150 rpm. The 10 g.L⁻¹ glucose condensed solution (25 mg glucose + 20 mL H₂O) was sterilized for 15 min at 121 °C and was added to the production medium before inoculation. After 6 h, the seed culture was fed into the 225 mL production medium, so that the production medium contains 10% inoculum. The
cultures were incubated at 150 rpm at 37°C. Several samples were withdrawn from each culture.

Sample was measured every one hour to check the cell growth and glucose concentration. Cell growth was monitored by measuring the optical density of sample at 600 nm (OD600). The glucose concentration, Cs (g.L\(^{-1}\)), was assayed using the 3,5-dinitrosalicylic acid method [7]. The maximum specific growth rate (\(\mu\)), Monod constant (Ks), and maximum specific growth rate were solved using the linearization of Monod’s formula, which was known as Lineweaver Burd formula [8].

After kinetic parameters are obtained, simulation of cellulase production through batch fermentation method and fed batch fermentation method is done with simulation software SuperPro Designer v9.0. Fermentor design and sizing is made with the same simulation software and manual calculation. Manual calculations are performed using reactor design conditions that are in accordance with the provisions in the literature [9, 10]

3. Results and Discussions

3.1. Determination of Escherichia coli BPPT-CC EgRK2 growth kinetic parameter

Fermentation results can be seen from Figure 1.

Figure 1. Growth curve of Escherichia coli BPPT-CC EgRK2 in different initial glucose concentration. (a) Cs0 10 g.L\(^{-1}\); (b) Cs0 1 g.L\(^{-1}\)

Figure 1 shows that bacteria growth in study A (initial concentration of glucose 10 g.L\(^{-1}\)) was much faster and higher than study B (initial glucose concentration 1 g.L\(^{-1}\)). This is happened due to the presence of adequate glucose content in study A. The addition of glucose is known to produce greater cell growth and biomass over time than not giving it at all [11]. From Figure 1, it is seen that the optical density of the cell indicates bacterial growth consisting of several phases, which are lag phase, exponential, stationary and death phase [12]. From Figure 1 it can be seen that at the 9th hour the bacteria growth is almost stopped because the glucose substrate concentrations are getting smaller and almost exhausted. This condition is true according to study that has been done by Monod, that glucose is the growth limiting substrate in bacteria fermentation [13].

Determination of growth kinetics parameter is done by the linearization of Monod equation, which is known as the Lineweaver-Burk equation [8]. The results of Lineweaver-Burk plot at initial glucose concentration 10g.L\(^{-1}\) are illustrated by Figure 2.
Figure 2. Lineweaver-burk plot to estimate Monod constants on *Escherichia coli* BPPT-CC EgRK2

From calculation of Figure 2, we get the results of $\mu_m$ and KS in this study are 1.694 h$^{-1}$ and 6,629 g.L$^{-1}$. The results obtained are quite high compared to kinetic parameters of E. coli on the glucose substrate found in other studies. Kinetic study of E. coli with Monod's equation was first done by Monod himself, and from the data interpolation, it was found that $\mu_m$ and KS are 1.35 h$^{-1}$ and 0.22 x 10$^{-4}$ M (Monod, 1949). Fogler in his book also stated that the E. coli kinetic parameters on glucose substrate that generally used are according to Monod’s study [8]. Poccia et al also estimated the growth model of E. coli JM 109 and BL 21 on multi-substrate media, and they obtained $\mu_m$ for JM 109 0.53 h$^{-1}$, while for BL 21 0.76 h$^{-1}$[14]. Study conducted by Kovarova showed E. coli ML30 on 500 mg.L$^{-1}$ glucose substrate have kinetic parameters $\mu_m$ 0.76 h$^{-1}$ dan KS 0.0328 mg.L$^{-1}$ [15].

From the above data it can be seen that $\mu_m$ and KS for *Escherichia coli* BPPT-CC EgRK2 on glucose substrate is much higher than *Escherichia coli* strain in general. This is due to the genetic recombination that has been done on *Escherichia coli* BPPT-CC EgRK2 which ultimately leads to high substrate consumption. In addition, the high value of $\mu_m$ may be due to the glucose content in fermentation medium. The article written by Schaechter states that *E. coli* grows faster on glucose media than other carbon and energy sources and can achieve doubling time of 20 minutes at 37 °C [16].

The value of KS obtained is 6,629 g.L$^{-1}$. High KS value indicates that in order to achieve its maximum growth rate, bacteria need a large amount of substrate, and this is not desirable in industrial production. Compared to other studies in literatures, this value is very high and abnormal, since KS values normally reside in the micromolar range, making it difficult to determine experimentally [17]. The high KS value also shows that bacteria are not efficient in using the substrate. There is also a possibility that substrate concentration used in this study is not optimum for *Escherichia coli* BPPT-CC EgRK2 growth, so that the KS value is too high; therefore it is necessary to do further research on optimum substrate concentration for growth of *Escherichia coli* BPPT-CC EgRK2.

3.2. Fermentor Design in Cellulase Production

In this study, a review of two types of fermentors is done, which are batch fermentor and fed batch fermentor. The operating conditions used in the fermentation process are in accordance with the optimum condition of *Escherichia coli* BPPT-CC EgRK2, which are temperature at 37°C and pH 7.0 [6]. Input and output balance data were obtained using SuperPro Designer v9.0 simulation. Calculation of the fermentor size was performed using the condition obtained from the literature [9, 10]. Here is
the detail of batch fermentor specification used in cellulase production.

Table 1. Batch Fermentor Specification

| Fermentor Batch | Type | Batch Amount |
|-----------------|------|-------------|
| Function        | Vessel where the bacteria growth take place |
| Handled material| Cell and fermentation media |
| Fluid volume (m³) | 81.96 |
| Fermentor volume (m³) | 102.44 |
| Mass Balance    | Component | Input (kg) | Output (kg) |
| Ash             | 18.63 | 18.65 |
| Biomass         | 98.78 | 3951.14 |
| Cellulase       | 8.00  | 8.00 |
| Cellulose       | 455.94| 455.91 |
| Fish powder     | 2483.10| 380.90 |
| Glucose         | 2266.09| 0.00 |
| Hemicelulose    | 874.07| 874.07 |
| Lignin          | 389.85| 389.85 |
| Sodium Hidroxide| 1.42  | 1.45 |
| Sodium Sulfate  | 72.32 | 72.34 |
| Sulphuric Acid  | 1.74  | 1.72 |
| Amonium Hidroxide| 402.67| 68.36 |
| Water           | 59039.78| 59566.97 |
| Air             | 13753.00| 13719.06 |
| Carbondioxide   | 0.00  | 356.98 |
| Total amount    | 79865.39| 79865.40 |

Design Specification

| Pressure (atm) | 1 |
| Temperature (°C) | 37 |
| Construction material | Stainless steel S316 |
| Vessel diameter (m) | 3.5 |
| Cylinder height (m) | 10.5 |
| Ellipsoidal height (m) | 0.6 |
| Cylinder thickness (in) | 1.2 |
| Ellipsoidal thickness (in) | 0.8 |
| Total fermentor height (m) | 11.7 |
| Hydrostatic pressure (bar) | 7.9 |
| Design pressure (bar) | 9.9 |

| Impeller type | 3 Rushton turbin |
| Agitation speed (rpm) | 150 |
| Impeller diameter (m) | 1.2 |
| Impeller width (m) | 0.2 |
In this research, fed batch fermentation simulation is also done on SuperPro Designer v9.0. SuperPro Designer provides the Fed-batch Fermentation feature as an option on batch fermentation if there is a simultaneous addition of material during the fermentation process. In this feature, the feed flow rate can be determined by specifying the flow rate of the stream (mass/volume) or determining the amount of material fed per cycle (mass/cycle). Nutritional feed on fed batch fermentation is composed of glucose as the main carbon source, fish powder (yeast extract), and water. The calculation of the feed flow rate was determined by the flow rate of streams on the Fed-batch tab. The constant volume flow rate of the feed is 320 L.h⁻¹ and begins to be added after fermentation runs 6 hours. Table 2 lists the detail of fed batch fermentor specification used in cellulase production.

| Table 2. Fed batch Fermentor Specification |
|--------------------------------------------|

### Fermentor Fed-batch

| Identification | Type | Fed Batch |
|----------------|------|-----------|
| Amount         | 1    |           |

| Function        |
|-----------------|
| Vessel where the bacteria growth take place |

| Handled material |
|------------------|
| Cell and fermentation media |

| Fluid volume (m³) | 84.188 |
|-------------------|--------|
| Fermentor volume (m³) | 105.24 |

### Composition (%)

| Component         | Input (kg) | Output (kg) |
|-------------------|------------|-------------|
| Ash               | 18.6       | 18.6        |
| Biomass           | 98.8       | 4630.5      |
| Cellulase         | 8.0        | 8.0         |
| Cellulose         | 455.9      | 455.9       |
| Fish powder       | 2910.1     | 412.3       |
| Glucose           | 2693.1     | 0.5         |
| Hemicellulose     | 874.1      | 874.1       |
| Lignin            | 389.8      | 389.8       |
| Sodium Hydroxide  | 402.7      | 1.4         |
| Sodium Sulfate    | 3.2        | 72.3        |
| Sulphuric Acid    | 70.5       | 1.7         |
| Ammonium Hydroxide| 1.7        | 5.6         |
| Water             | 59894.1    | 60520.5     |
| Air               | 13753      | 13712.2     |
| Carbon dioxide    | 0          | 424.2       |
| Total Amount      | 81573      | 81573       |

| Design specification |
|-----------------------|
| Temperature (°C)      | 37         |
| Construction material | Stainless steel S316L |
From Table 1, it is known that the batch fermentation method produces 3,951.14 kg of biomass. On the other hand, the fermentation by fed batch method is capable of producing 4,630.5 kg of biomass. It can be concluded that by using simulations, the fed batch fermentation method produces 18% more biomass (cell) than in batch fermentation.

This addition occurs because basically the main advantage of fed batch fermentation method is to add nutrients when the amount of nutrients in the media starts to run out. In addition, fed batch fermentation can extend the long log phase of bacterial growth, resulting in rapid cell production over long periods of time. Because of this, the fed batch fermentation method is suitable for microbial production using Escherichia coli, since Escherichia coli fermentation time is short due to its rapid doubling time, which is about 15-20 minutes at 37 °C [18].

From the specifications shown above, it can be seen that the two methods of fermentation do not have much effect on the volume and size of the fermentor. In the batch fermentation method, the fermentor volume required with 20% safety factor is 102.44 m$^3$; while for fed batch fermentation method, the required fermentor volume with a 20% safety factor is 105.2 m$^3$. In other words, the two methods of fermentation have a difference in volume size of approximately 3 m$^3$. This volume difference occurs because in the fed batch fermentation method there is constant addition of nutrient feed. This contributes to the addition of fluid volume in the fermenter. It is for this reason that the fermentor size determination should also take into account the possible increase in volume in order for the fermentor to avoid overflow in fermentor.

In the fermentor size calculation, the size of the fermentor tank and its stirrer must be adjusted to the process and type of organism handled inside [9]. In both fermentors, the ratio of fermentor diameter to height is 1:3, which is the optimum ratio for microbial applications on an industrial scale [9]. This ratio provides good aeration and a large area for heat exchange within the media. Both fermenters also given impellers to provide better agitation. This is happened because the vessel high can become very critical on the large fermentor vessel. The high size of the vessel will cause high hydrostatic pressure at the bottom of the vessel, and it can affect the life of cells and products it produces.
4. Conclusions

Monod growth kinetic parameters of *Escherichia coli* BPPT-CC EgRK2 in glucose substrate on this study are 1.694 h\(^{-1}\) and 6.629 g.L\(^{-1}\). Total biomass amount produced in batch fermentation is 3951.14 kg/batch and total biomass amount produced in fed batch fermentation is 4676.48 kg/batch. In large scale cellulase production, fermentor volume needed is 102.44 m\(^3\) for batch and 105.23 m\(^3\) for fed batch. Fed batch fermentation can produce more cellulase than usual batch fermentation. Therefore, fed batch fermentation is the better fermentation method in producing cellulase in a large scale.

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5. References

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