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Bioethanol Production from Sugarcane Molasses by Fed-Batch Fermentation Systems Using Instant Dry Yeast

Agustin Krisna Wardani[1]*, Cinthya Putri Utami[1], Mochamad Bagus Hermanto[2], Aji Sutrisno[1], and Fenty Nurtyastuti[1]

[1]*Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang 65145, Indonesia*
[2]*Department of Bioprocess Engineering, Faculty of Agricultural Technology, Universitas Brawijaya, Malang 65145, Indonesia*

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Bioethanol has recently attracted much attention as a sustainable and environmentally friendly alternative energy source. This study aimed to develop a potential process for bioethanol production by fed-batch fermentation using instant dry yeast. To obtain the highest cell growth, we studied the influence of the initial sugar concentrations and pH of sugarcane molasses in batch fermentation. The batch system employed three levels of sugar concentrations, viz. 10%, 15%, 20% (w/v), and two levels of pH, 5.0 and 5.5. The highest cell growth was achieved at 20% (w/v) and pH 5.5 of molasses. The fed-batch system was then performed using the best batch fermentation conditions, with a molasses concentration of 13% (w/v) which resulted in high ethanol concentration and fermentation efficiency of 15.96% and 89%, respectively.

Keywords: **Batch fermentation, bioethanol, fed-batch fermentation, instant dry yeast, sugarcane molasses**

Introduction

Energy security is a significant issue of the world. The increasing energy consumption has considerably dropped the fuel resource and the total global petroleum consumption keeps increasing due to high energy consumption use [1]. Bioethanol is usually produced from plant materials such as corn, sugar cane, and sugar beet. However, suppose edible food crops for humans and livestock are exploited as raw materials, an ethical problem might emerge due to the rise of food prices for competitive demands [2]. Therefore, the use of non-food biomass which is affordable and abundant in bioethanol production is more desirable.

Specifically, sugar cane molasses biomass has appeared as a promising alternative for bioethanol production. Molasses, a by-product of the sugar industry, contains a significant quantity of sugar, 40 to 50% (w/v), and ash content of 5 to 15%. The high sugar level and low prices make molasses a promising alternative as a fermentation substrate for bioethanol production [3]. The fermentation process requires fermentative microorganisms to convert sugar to ethanol. *Saccharomyces cerevisiae* strains are commonly used in the food industry and the laboratory because they are easy to grow with a high tolerance of up to 20% (v/v) towards ethanol [4]. However, there are disadvantages to using pure cultures of *S. cerevisiae*, such as required inoculum preparation and high risk of contamination. Cultures must be grown to reach the desired number of a cell by inoculation in a liquid medium [5]. An alternative method used instant dry yeast to start ethanol fermentation to solve these problems. The application of instant dry yeast in bioethanol production can simplify the operation process as it can be directly used on medium fermentation and

***Corresponding author**
Phone: +62-812-5253-3560, Fax: +62-341-568917
E-mail: agustinwardani@ub.ac.id

reduce the risk of bacterial contamination [6]. The other advantages of instant dry yeast are remarkable tolerance to high sugar concentrations, high growth rate, and time saving for fermentation [7]. Instant dry yeast can produce ethanol more optimally because it consists several selected superior strains from *S. cerevisiae,* each of which has its own superior characteristics [8]. Instant dry yeast is commercially produced under control. Several superior strains are isolated and grown in certain medium (generally molasses) before use for fermentation. Some of these superior strains are selected because they have stable physiological characteristics such as fast growth rate, high cell yields, and convenient storability [9]. Preliminary research using molasses of 20%, 25%, and 30% Brix found that the ethanol content by yeast ranged from 4–6%. However, when using instant dry yeast, the ethanol concentration was higher, ranging from 5–9%. In addition, a more diverse cell shape was found for the yeast starter, which was used by industry (possible contaminants), while instant dry yeast culture had a more uniform cell hape [5]. Thus, this study used instant dry for ethanol production.

Most industrial fermentation process is operated in batch fermentation because it is simple to operate; where all carbon source and media components are added in bulk at the start of the fermentation and then run until the carbon source is depleted. This system is simple to operate, yet, it requires extended downtime for batch turnaround. It is also inefficient with changing substrate concentrations and not allowing control over the yeast growth rate [10]. An alternative is fed-batch fermentation, where the first fermentation stage is operated in batch mode with a bulk carbon source to promote biomass accumulation. Once this bulk is depleted, feeding begins to supply the system with a carbon source for product formation and biomass growth and maintenance [11]. During the fermentation process, cell growth inhibition can be induced by mineral or substrate concentration of molasses. The mineral content is in the form of Ca, K, and Mg. The sugar concentration in sugarcane molasses must also be considered in the fermentation process. During fermentation, yeast converts sucrose into reducing sugar through the enzyme invertase. The effect of calcium ions on ethanol production from sugarcane molasses by yeast shows that metal ion content could inhibit the enzyme activity of invertase. The strategy to reduce mineral levels, especially calcium, in sugarcane molasses is decalcification using H_2SO_4 [12]. In this study, we investigated the effect of pH and substrate concentration of decalcified molasses on yeast growth. After obtaining the best molasses concentration and pH for yeast growth, they were applied in fed-batch fermentation for optimal ethanol production.

Materials and Methods

Microorganism

Commercial instant dry yeast was used to produce ethanol. The instant dry yeast of unicellular organisms *S. cerevisiae* were purchased from PT. Sangra Ratu Boga (Indonesia).

Molasses pretreatment

Molasses were pretreated with sulfuric acid prior fermentation. Molasses media were prepared by diluting with water in a ratio of 1:1 with an initial pH of 5.1. Concentrated H_2SO_4 solution (96.1%) was added until the pH reached 3.9, then heated to 95℃ for 10 min. The precipitates were then removed by filtration. The pretreated molasses were subsequently adjusted using NaOH to achieve a pH of 5.0 and 5.5. Sugar concentration was adjusted by additional water to obtain sugar concentrations of 20%, 25%, and 30% Brix. Some essential nutrition was added to ensure the proper yeast growth during ethanol fermentation, including 0.1% (w/v) yeast extract and 0.1% (w/v) peptone. Then, the medium was autoclaved at 121℃ for 15 min.

Fermentation conditions

The fermentation process was carried out in two stages. In the first stage, batch fermentation was performed to determine the best molasses concentration and pH. In batch fermentation, three sugar concentrations were used, which were 10%, 15%, and 20% (w/v). Furthermore, two levels of pH, 5.0 and 5.5, were applied during fermentation. Inoculation was made using 4% of instant dry yeast. In the second stage, fed-batch fermentation was performed using the best condition of batch fermentation in terms of yeast growth. Fermentation was carried out in a water bath shaker at 30℃, 110 rpm for 72 h. Analysis of fermentation was conducted for sugar concentration, optical density, total cells, pH and

ethanol concentration.

Analytical methods

The cell growth was measured using a UV-visible spectrophotometer (UV mini, Shimadzu, Japan) at OD600nm. The total solid content in the medium was measured by a Brix refractometer (Indonesia). The total sugar was measured by phenol sulfuric acid based on previously described methods [6]. To measure the total cell, we used total plate count method. Meanwhile, to measure the calcium concentration, we employed analytical method of AAS (Atomic Absorption Spectroscopy) with the following preparations: one gram of the sample was dissolved with 6 ml of HNO_3 (65%) and 2 ml of H_2O_2 (30%). It was then dried by increasing the temperature from 40–50℃ to 140℃. The residue was diluted to 10 ml with ionized water, and the calcium concentration was measured using AAS [12]. The ethanol concentration was measured by Gas Chromatography (GC-2010, Shimadzu, Japan) with the following preparations: samples (fermentation broth) were centrifuged by a mini centrifuge (MC-12, Benchmark, USA) at 10.000 rpm. The supernatant was injected into a Restek Rtx-Wax GC column (USA) at 80℃. Helium was used as carrier gas with a flow rate of 30 ml/min, and eluted compounds were detected by a flame ionization detector (FID). Hydrogen was used as fuel gas, with a flow rate of 40 ml/min, and air at a flow rate of 400 ml/min. Secondary butanol was used as an internal standard.

Kinetics of batch and fed-batch fermentation

The specific growth rate (μ) is not constant depending on the physical and chemical environmental conditions. The maximum growth rate value (μ_{max}) is achieved when the supply of substrates and nutrients are still excessive and the concentration of metabolic substances that inhibit growth is remains low and can be expressed in Eq. (1) as follows:

$$\mu_{max} = \frac{\ln(X - X_o)}{\Delta t} \quad (1)$$

The yield coefficient of living cells to carbon sources is expressed as Yx/s (Eq. (2)). The conversion coefficient of nutrients in the substrate into products at a certain time is expressed in Yp/s (Eq. (3)). Whilst the conversion coefficient of nutrients in the substrate into products in a certain period is expressed as Yp/x (Eq. (4)).

$$Y_{x/s} = \frac{\Delta X}{\Delta S} = \frac{(X - X_o)}{(S_o - S)} \longrightarrow (X - X_o) = Y_{x/s}(S_o - S) \quad (2)$$

$$Y_{p/s} = \frac{\Delta P}{\Delta S} = \frac{(P - P_o)}{(S_o - S)} \longrightarrow (P - P_o) = Y_{p/s}(S_o - S) \quad (3)$$

$$Y_{p/x} = \frac{\Delta P}{\Delta X} = \frac{(P - P_o)}{(X_o - X)} \longrightarrow (P - P_o) = Y_{p/x}(X_o - X) \quad (4)$$

Results and Discussion

Characteristic of molasses with and without H_2SO_4 pretreatment

Yeast converts sucrose into reducing sugar by utilizing the enzyme invertase during fermentation. This study used pretreatment with H_2SO_4 or decalcification of molasses to reduce calcium levels. It is commonly acknowledged that the effect of calcium ions on ethanol production from sugarcane molasses by yeast could inhibit the enzyme activity of invertase. A sulfuric acid pretreatment process reduces the amount of calcium, ash, and other impurities [13]. Table 1 shows the characteristic of molasses with and without pretreatment using H_2SO_4. The data demonstrated that all components in the pretreated molasses with acid pretreatment were lower than those without. Even, the calcium content reduction in molasses significantly decreased from 0.64% to 0.02% after adding H_2SO_4. It occurs due to the separation process of minerals from molasses during 24 h incubation. The presence of ion metal content, such as copper, calcium, and potassium, can inhibit the activity of the invertase enzyme secreted by yeast from converting sucrose to reducing sugar [20]. Adding H_2SO_4 can reduce mineral levels, mainly calcium, in sugarcane

Table 1. Characteristic of sugar cane molasses.

Parameter	Without pretreatment	With pretreatment
Total soluble solid (% Brix)	80.5 ± 0.5	44.5 ± 0.5
Total sugar (%)	54.08 ± 0.16	45.64 ± 0.10
Reducing sugar (%)	19.69 ± 0.17	15.44 ± 0.11
Total ash (%)	7.46 ± 0.03	4.19 ± 0.03
Mineral (Ca) (%)	0.64 ± 0.15	0.02 ± 0.20

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molasses. The SO_4^{2-} ion from sulfuric acid will bind the positive ions from metal in the sample to form calcium salt deposits.

Determination of sugar concentration and pH molasses for fed-batch fermentation

Fermentation medium conditions, such as sugar concentration and pH, are fundamental in the fermentation process to obtain optimal results. If they fit the yeast growth, the fermentation efficiency will increase [16]. In this study, the best substrate concentration and pH obtained in a batch fermentation were then used to achieve higher ethanol yields in fed-batch fermentation. The best substrate concentration and pH were drawn from the highest yeast growth. The growth profile of instant dry yeast during batch fermentation with variations of molasses concentration and pH is presented in Fig. 1.

Fig. 1 shows that yeast cells (10.44 log CFU/ml) reached the highest growth rate in pH of 5.5 and molasses concentration 20%. These results indicated that the higher the concentration of molasses, the higher the growth of yeast cells. The high sugar concentration in molasses can increase nitrogen utilization by yeast for growth and maintenance over cell performance in enzyme formation for ethanol production [17]. Adequate nitrogen in molasses is essential for synthesizing amino acids and proteins, which will be used for yeast physiology [18]. Another study proved that the growth rate of yeast increased with additional molasses in the fermentation medium [19].

In all conditions of fermentation, the number of the cells decreased after 18 h of fermentation because the yeast could no longer multiply, and some cells had died. At this stage, environmental density is reduced due to the conversion of sugar to ethanol, so the cell concentration decreases [20]. The pH of the fermentation medium is also a factor for the increase of cell growth, so the yeast enzymes work optimally. The cell reached the highest growth at pH 5.5 of medium. If the pH of the fermentation medium is low, the fermentation process would not be optimal because it interfered with the metabolic process of yeast [12]. Based on the growth profile of instant dry yeast (Fig. 1), the fermentation conditions with molasses concentration of 20% (w/v) and a pH of 5.5 were then adopted to carry out fed-batch fermentation for ethanol production.

Batch fermentation of bioethanol production

Batch fermentation was performed in a water bath shaker at 30℃ for 72 h. The profile of ethanol fermentation in a batch system is presented in Fig. 2. The substrate was available in the form of sugar concentration, which was initially 20% (w/v), then decreased to 2.77% (w/v) during fermentation (Fig. 2). It was used by yeast growth, reproduction, and production of metabolites in the form of ethanol. The decrease in sugar continued to occur during the fermentation process because in the

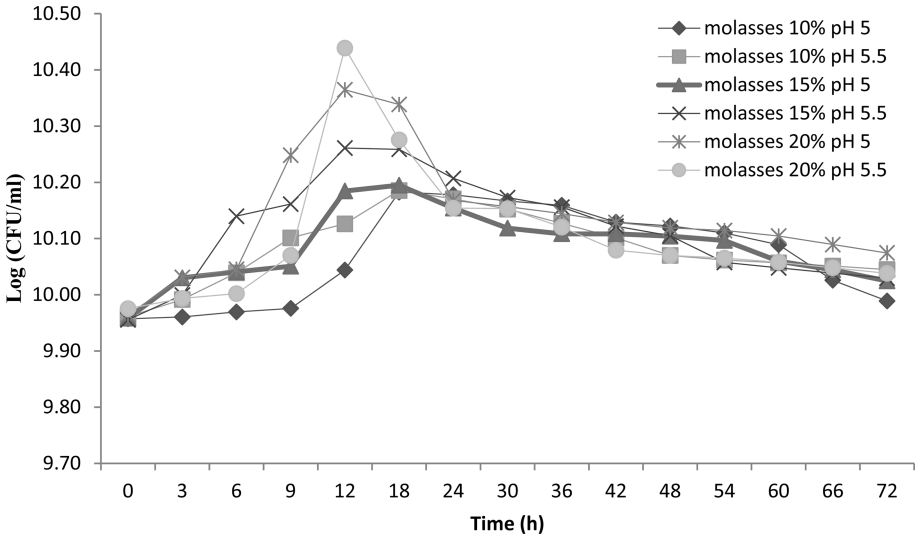

Fig. 1. Instant dry yeast growth profile (log CFU/ml) under batch fermentation using molasses as a substrate.

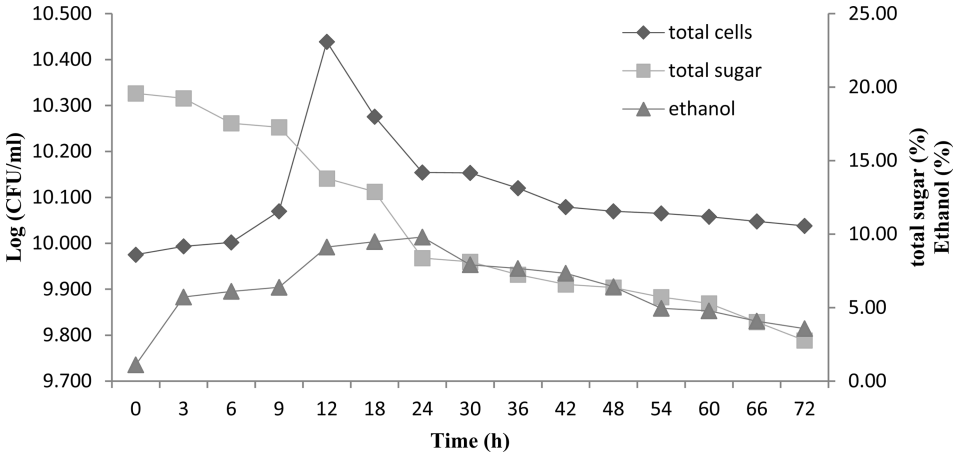

Fig. 2. The profiles of cell number of instant dry yeast (log CFU/ml), total sugar (%), and ethanol concentration (%) under batch fermentation.

batch system, there was no addition of a new substrate [22]. The concentration of sugar medium fermentation is substantial for ethanol production. If the medium contains too high sugar concentration, the fermentation process may not run optimally because it takes time for fermentation, loss sugar content, and the fermentation efficiency level is lower. The fermentation efficiency will increase if the medium fermentation is suitable for yeast [20].

The sugar concentration in the fermentation medium at 30 h was very low (8.1%), followed by a decrease in the ethanol content. It indicated that the extremely low total sugar could no longer meet the yeast's needs to produce ethanol. The high number of yeast, 10.44 log CFU/ml was observed at 12 h (exponential phase). However, after 12 h of fermentation, the total cell decreased to 10.04 log CFU/ml. The highest ethanol production was reached during 24 h of fermentation, which was 9.80%. Unfortunately, the batch fermentation process is a lack in that the concentration of ethanol produced during fermentation will accumulate and become toxic for the yeast. The accumulation of toxic dissolved products will decrease slowly and even stop the growth and production of ethanol from yeast [23].

Fed-batch fermentation of bioethanol production

In the fed-batch fermentation system, a new substrate is added regularly into the fermentation medium with a

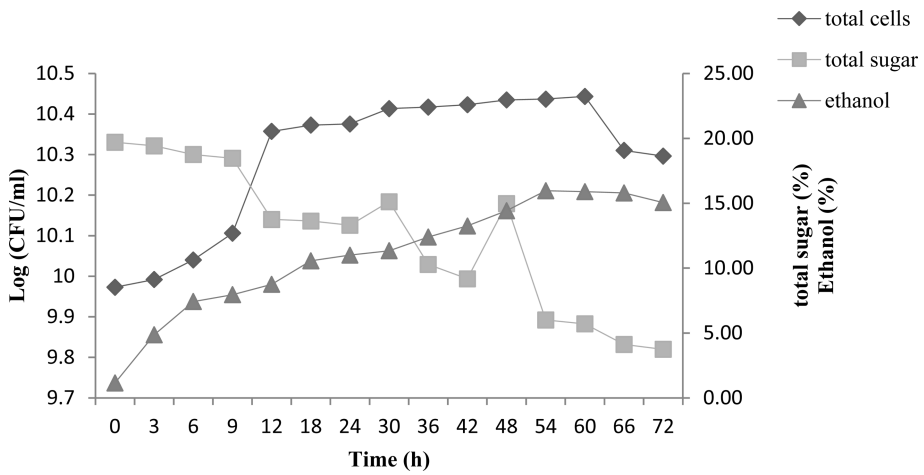

Fig. 3. The profiles of cell number of instant dry yeast (log CFU/ml), total sugar (%), and ethanol concentration (%) under fed-batch fermentation.

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low concentration and particular volume during fermentation. The main advantages of the fed-batch fermentation operation system are the control of microbial growth rate, and it reduces the toxic effects of the media components [13]. Fig. 3 demonstrated that yeast growth in a fed-batch system was in the exponential phase at 12 h of fermentation with a total cell of 10.36 log CFU/ml. The new substrate of 13% (w/v) molasses was added at 12 h, 30 h, and 48 h of fermentation, causing the maximum total yeast to be achieved longer (extending the exponential phase).

The yeast cell began to decrease in number after 60 h fermentation. Also, the yeast population decreased in the substrate yet increased in ethanol production. It reached maximum ethanol production at 54 h of fermentation, which was 15.96%. The ethanol concentration produced in the fed-batch fermentation was higher than that from the batch system. Due to additional new media in the fed-batch system to add nutrients for yeast to metabolize and produce more dehydrogenase enzymes, the conversion of sugar into ethanol was higher [4]. Ethanol began to decrease at 66 h of fermentation. It occurred because the yeast population and sugar content decreased. The availability of sugar as a carbon source for yeast cells was lower, so it is no longer sufficient for yeast to metabolize. In addition, yeast has come to the death phase, so yeast cells have died and lost the ability to grow. Suppose the yeast is no longer able to produce the dehydrogenase enzyme, it will decrease the conversion of sugar into ethanol and reduce the concentration of ethanol production [24].

Kinetics of bioethanol production during batch and fed-batch fermentation

The process of cell growth is extremely complex, including entering essential nutrients from the environment into cells, converting nutrient materials into energy, and various essential constituents for their proliferation. The efficiency of bioethanol production can be evaluated by three parameters: yield, productivity, and final product concentrations. Ethanol yield can refer to metabolic or process yield, calculated as ethanol produced based on consumed sugars [25]. The kinetics of batch and fed-batch fermentation are presented in Table 2. The maximum specific growth rate for batch fermentation (0.037) was higher than the fed-batch (0.025), Yx/s in batch fermentation (0.058) was lower than fed-batch (0.172), Yp/s of batch fermentation (0.775) was lower than fed-batch (0.801). The yield of product formation to cells (Yp/x) of batch fermentation was lower than the fed-batch, which was 2.529 and 4.660, respectively. The efficiency of using substrate (So-S)/So in the batch system was lower than that in the fed-batch, which was 86% and 89%, respectively. Yx/s, Yp/s, Yp/x, and (So-S)/So values in fed-batch fermentation were higher than those in batch fermentation, because when a new substrate was added to the fermentation medium, the source of food intake for yeast increases, so yeast would produce or carry out the respiration process for ethanol and cell formation. As for the maximum specific growth (μ_{max}), the highest was in the batch system. In batch fermentation conditions, the yeast used more energy for cell growth than to produce ethanol. The results of this study correspond with those of previous study for bioethanol production that fed-batch fermentation is superior to batch fermentation. The ethanol produced by *S. cerevisiae* using *Prosopis juliflora* was 3.5% under batch fermentation, while 5.3% under fed-batch fermentation [26]. Another study showed that fed-batch fermentation of sorghum juice by *S. cerevisiae* provided better performance for ethanol production (12%) than batch fermentation (8%) [28]. As a comparison, our research shows that producing bioethanol from molasses using a fed-batch fermentation system by instant dry yeast achieved higher ethanol (15.96%), because molasses, as a raw material for ethanol, contains a high sugar content of 50–60% (w/v) sugar compared to other raw materials [2]. Furthermore, the use of instant dry yeast is very beneficial because it exhibits a good performance in converting sugar into ethanol, as in the high results of the fermentation efficiency, 89.9%.

Table 2. Batch and fed-batch fermentation kinetics.

Parameter	Batch fermentation	Fed-batch fermentation
μ_{max} (h^{-1})	0.037	0.025
Yx/s (g/g)	0.058	0.172
Yp/s (g/g)	0.775	0.801
Yp/x (g/g)	2.529	4.660
(So-S)/So (%)	86	89

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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