Optimization of Citric Acid Production by Utilizing Rice Husk Waste as a Substrate Using Submerged Fermentation

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Abstract. Citric acid is an organic acid and is highly used as a commercial product. The production of citric acid in Indonesia has not met its demand, a cheap and easy method of obtaining raw materials to boost its production is required. Submerged fermentation using *Aspergillus niger* is a prospective method of citric acid production. Microorganisms need substrate as a carbon source to produce citric acid. Rice husks have the potential to be used as substrate to produce citric acid because they contain high cellulose (28.6–43.3%). This research aims to obtain the optimum fermentation conditions of producing citric acid. Variations in the study were methanol concentration (1–3% v/v), sucrose concentration (0–10% w/v), substrate concentration (15–30% w/v), and agitation speed (100–400 rpm). The variations were determined by a statistical approach of Response Surface Methodology. During the fermentation process, pH and citric acid concentration were measured. The pH and citric acid concentration data was analyzed using Minitab. The optimal conditions of producing citric acid using rice husks were obtained. They were substrate concentration 22.42% w/v, sucrose concentration 10% w/v, methanol concentration 3% v/v, and agitation speed 400 rpm. These optimal conditions resulted in a citric acid concentration of 19.12 g/L in pH 2.34 in 5-day fermentation.

Keywords: Aspergillus niger · Central composite design · Citric acid · Rice husk · Waste utilization

1 Introduction

Citric acid has been widely used in various industries such as in food and beverages industries (60%), pharmaceutical and detergent industries (30%), and in cleaning products industries, textile dye, and cosmetics [1]. However, there is a significant gap between citric acid production and its demand in Indonesia. There is a high demand for citric acid in Indonesia, which is 208,000,607 kg per year. Meanwhile, its annual production only reaches 7,881,624 kg per year [2].

Citric acid can be produced through citrus fruit extraction, chemical synthesis, and fermentation [3]. Producing citric acid through citrus extraction has started to be less

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popular because growing market for citrus has led to citrus trade monopoly and citrus farming land is inadequate [4]. On the other hand, chemical synthesis method is not fully perceived to be reliable due to safety impact of the product. Fermentation is considered a more prospective method of producing citric acid. Submerged fermentation is the most applied technique for citric acid production. About 80% of global citric acid production is through submerged fermentation [1]. This technique has a number of advantages: high microbes and yield productivity, easy temperature and humidity control, low risk of contamination, and not easily affected by changes in medium composition [5].

In citric acid production through fermentation, *Aspergillus niger* has been highly used as it enhances the yield concentrate and it is low cost [6]. Microorganisms need substrate as a carbon source to produce citric acid. With an abundance of rice husks in Indonesia, particularly in Toba Regency, North Sumatera, they can be utilized as a substrate in citric acid fermentation. Rice husks contain relatively high cellulose (28.6–43.3%) and hemicellulose (12.0–29.7%) [7–10]. According to a report of Statistics Indonesia in North Sumatera in 2020, rice production in Toba Regency was 106,168.3 tons. It indicated that rice husk production reached 21,233.6 tons or about 20% of the rice. The abundance of rice husks indicates great potential to produce citric acid through fermentation because rice husks' composition can be used as a substrate in the fermentation. In addition, they are cheap and easily collected. Moreover, rice husks' utilization has not been explored optimally. Therefore, this study aimed at optimizing submerged fermentation conditions of rice husks in citric acid production.

2 Materials and Methods

2.1 Preparation of Rice Husk Substrate

Rice husks were collected from the local mills, which were husks from local rice in Toba Region. Fresh rice husks were cleaned and then oven-dried at a temperature of 55 °C for 24 h. The dried rice husks were mechanically pretreated using grinder IKA MF 10 with rotor speed of 1500 rpm and sieve size of 0.5 mm. The pretreatment was continued with chemical pretreatment using NaOH 1% and H_2SO_4 1% respectively to maximize delignification. The pretreatment procedure followed the procedure described by [11, 12]. A solution of NaOH 1% was added to the rice husk ash with volume ratio of 1:10. The rice husk ash was then incubated in water bath at 85 °C for 1 h. The sample was filtered and washed using distilled water until it reached neutral pH level of 7. After that, it was oven-dried at 105 °C. The rice husks from alkaline pretreatment were then pretreated in acid pretreatment using H_2SO_4 1% with the ratio of 1:2.8. Next, they were heated in an autoclave at 121 °C for 75 min. The rice husk pulp was filtered to separate it from the water contained in it. NaOH 4% with the ratio of 1:1.5 was added into the pulp. Then, the pulp was heated with an autoclave at 121 °C for 30 min. The solid phase was separated and washed with water a few times. After the last washing, the pH level the solid phase was measured. If the pH level was neutral, the rice husks would be processed through hydrolysis. The enzymatic procedure followed the procedure performed in a study done by [12]. Distilled water was added into the rice husk pulp from the pretreatment with the volume ratio of the pulp and distilled water was 1:5. Then, a solution of H_2SO_4 10%

as a buffer was slowly added until the pH level was 5.4. Next, the pulp was heated in an oven at 100 °C for 35 min.

The next step was to cool the pulp until it reached room temperature. Then, cellulase enzyme with 10% total enzyme fraction whose activities were 10,000 U/mL (5 mL enzyme per 50 g of biomass) was added. The pulp was stirred and incubated for 24 h with agitation of 150 rpm at 45 °C. Enzyme activity was stopped using an autoclave at 121 °C. The result of the hydrolysis could be used directly in the fermentation.

2.2 Cultivation of Aspergillus Niger

Aspergillus niger was obtained from Laboratory of Microbiology, SITH, ITB. *A. niger* was reproduced in Potato Dextrose Agar (PDA) and incubated at 37 °C for 3 days. A standard curve was generated by making 8 variations in *A. niger* inoculum dilutions using NaCl 1%. The absorbance was measured using a spectrophotometer UV-Vis at wavelengths of 600 nm. Next, the sample was centrifuged at a speed of 1200 rpm for 5 min and oven-dried at 60 °C for 24 h to obtain dry cell weight. A linear regression curve was generated by plotting the absorbance value versus cell concentration (g/mL). Then, the linear equation was solved. A growth curve was created by inoculating 3 inoculation loops of *A. niger* in Yeast Peptone Glycerol (YPG) medium. Sampling was done every 2 h. The absorbance was measured at wavelengths of 600 nm. Sampling was done until the growth of *A. niger* reached stationary phase. Data of the absorbance was converted to cell concentration (g/mL) using the linear equation obtained from the standard curve. Next, a growth graph of cell concentration versus time. Based on the growth curve, the inoculum age in exponential phase used in the fermentation was 36 h, or with absorbance 0.39 at wavelengths of 600 nm and concentration of 27.5 g/mL.

2.3 Generating the Standard Curve of Citric Acid

Citric acid standard curve was generated 8 variations in citric acid dilutions using distilled water with working volume of 10 mL and citric acid concentration of 10 mg/mL. The absorbance value was measured using a spectrophotometer UV-Vis at wavelengths of 407 nm. Then, a linear curve of absorbance value versus citric acid concentration (mg/mL). Finally, the linear equation was solved.

2.4 Preparation of Fermentation Medium

The working volume in this study was 200 mL with the following compositions: NH_4NO_3 0,25%, KH_2PO_4 0,1, $MgSO_4$ 0,025% [13] and rice husk substrate 15–30% b/v, sucrose 0–10%b/v, and methanol 1–3% v/v. The fermentation medium prepared was then sterilized in an autoclave at 121 °C.

2.5 Submerged Fermentation

As much as 5% *A. niger* inoculum of the total fermentation working volume was inserted into the fermentation medium. The medium was homogenized with variations in stirring speed of 100–400 rpm and incubated at 30 °C for 5 days.

2.6 Citric Acid Analysis

Sampling of the fermentation medium was done every 24 h for 5 days. The sample underwent centrifugation at rotating speed of 3000 rpm for 20 min to collect citric acid which was produced by *A. niger* in the supernatant. As much as 3 mL supernatant was measured for its pH level and another 3 mL was used to analyze the concentration of citric acid. The citric acid concentration was analyzed using pyridine-acetic anhydride method. 1.3 mL pyridine was added to 3 mL supernatant to be homogenized. Next, 5.7 mL acetic anhydride was added to the supernatant and then homogenized by using vortex. The next step was to incubate the mixture for 30 min at 32 °C until light yellow in color. The concentration of citric acid was measured using a spectrophotometer UV-Vis at wavelengths of 407 nm [14]. The absorbance value recorded was substituted in the linear regression of the citric acid standard curve to know the citric acid concentration in the sample. The concentration unit was %v/v.

2.7 Experiment Variations

Experiment variations of the rice husk substrate concentration, sucrose concentration, methanol concentration, and stirring speed were determined by applying a statistical approach namely Central Composite Design-Response Surface Methodology (CCD-RSM) (Table 1). Data on citric acid concentration for each parameter was analyzed using Minitab to obtain optimum parameter conditions to produce maximum citric acid.

3 Results and Discussion

Citric acid can be produced through fermentation using *Aspergillus niger.* The optimum conditions of substrate concentration, sucrose concentration, methanol concentration, and agitation speed may affect citric acid production by *Aspergillus niger*. Based on the analysis using Minitab (Fig. 1) on measured pH level and citric acid concentration obtained from the 19 variations of the fermentation, the optimum conditions were as following: rice husk substrate concentration is 22.42% w/v, sucrose concentration is 10% w/v, methanol concentration is 3% v/v, and agitation speed is 400 rpm. Maximum concentration of citric acid yielded on day 5 of the fermentation is 19.12 g/L.

Figure 2 illustrates citric acid concentrations and pH levels during the 5-day fermentation. It can be seen that the highest citric acid concentration was 19.12 g/L, while the lowest pH value was 2.34.

In this study, there were variations in substrate concentration with the range of 15–30% w/v. The optimum concentration recorded was 22.42% w/v. [15] conducted a study to investigate variations in substrate concentration in citric acid production using banana peel waste that contains high cellulose and hemicellulose. The citric acid production increased until the substrate concentration reached 25% w/v. [16] also completed a study to examine effects of corn cob substrate concentration on citric acid production using *A. niger.* Corn cobs are rich in lignocellulose. In their study, [16] compared the substrate concentrations and they found that the optimum substrate concentration to produce citric acid was 24% v/v. It was also found that higher substrate concentration could lead to

Table 1. Experiment variations based on central composite design method

Run	Substrate (%w/v)	Sucrose (%w/v)	Methanol (%v/v)	Stirring speed (rpm)
1	15	0	1	100
2	15	0	3	100
3	15	10	1	100
4	15	10	3	100
5	30	0	1	100
6	30	0	3	100
7	30	10	1	100
8	30	10	3	100
9	15	0	1	400
10	15	0	3	400
11	15	10	1	400
12	15	10	3	400
13	30	0	1	400
14	30	0	3	400
15	30	10	1	400
16	30	10	3	400
17	22.5	5	2	250
18	22.5	5	2	250
19	22.5	5	2	250

decreasing citric acid yield. The decrease could be caused by low water content in the high concentration substrate that prevented microorganisms' growth. High substrate concentration in the fermentation medium will lead to large concentration gradient on intracellular fluid. It may cause the fluid to flow outside of the cell and finally it may result in cell crenation. In a substrate with too low concentration, the water content in a fermentation medium will increase and it may lead to less nutrition in the medium which can inhibit microorganisms' growth [16, 17].

It has been found that the optimum concentration of sucrose, an additional carbon source, is 10% w/v. According to [18], not every substrate used in producing citric acid through fermentation requires additional carbon. A substrate with a reducing sugar about 30% does not need sucrose as an additional carbon source. In citric acid production using rice husks, it is necessary to include an additional carbon source in the process because rice husks contain a reducing sugar with low concentration. A carbon source in a medium for citric acid fermentation is one of the factors affecting the citric acid yield. Based on the studies conducted by [19] and [20], the carbon source that can produce optimum citric acid is sucrose. Sucrose is a type of carbon that can enhance *Aspergillus*

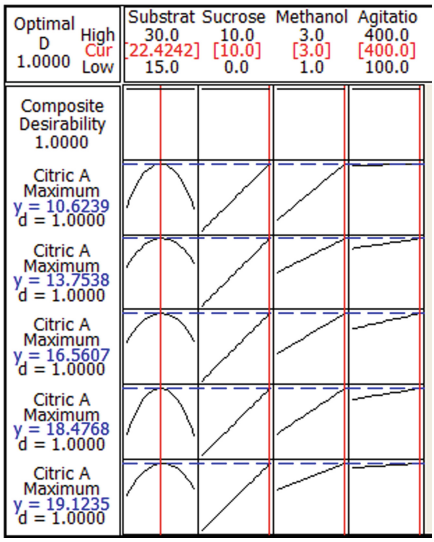

Fig. 1. Analysis result on measured pH level and citric acid concentration obtained from the 19 variations of fermentation conditions.

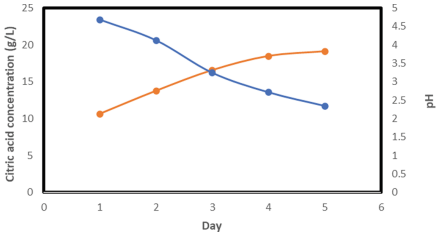

Fig. 2. Concentrations of citric acid and pH value during the citric acid fermentation.

niger activities because *Aspergillus niger* contains extracellular invertase enzyme that has strong bond in mycelium capable of working actively at low pH and hydrolyzing sucrose into glucose and fructose quickly [21].

In addition to the types of a carbon source, the concentration of a carbon source used in a medium affects citric acid yield as well. A carbon source with too high concentration may cause an accumulation of fermentation by-product such as oxalic acid and an increase in viscosity of the fermentation medium due to overgrowth of mycelium that finally may decrease dissolved oxygen in the medium [22]. Low dissolved oxygen may result in mycelium shrinkage and oxalic acid accumulation during fermentation [5].

Methanol is the best type of alcohol to enhance citric acid yield [23]. Several studies were conducted to investigate effects of the types and concentration of alcohol on citric acid production [24–26]. In the studies, the types of alcohol used in the fermentation were methanol, ethanol, butanol, isopropyl alcohol. It was found that the most optimum citric acid yield was with the addition of methanol with different concentration depending on the substrate types. According to [27], the addition of methanol to a fermentation

medium may increase citrate synthase activity and decrease aconitase activity as much as 75%, so the production of citric acid can be enhanced. The addition of methanol to produce citric acid is to neutralize negative impacts of metal ions [28]. [29] said that methanol would disrupt the structure of cell membranes composed of lipid and protein so that the cell's permeability became higher and therefore it enabled the metal ions to permeate the cell. Further, they added that alcohol with longer hydrocarbon chains such as ethanol, isopropyl alcohol, and butanol would damage the cell membranes. However, the addition of alcohol with higher concentration may interfere with the production of citric acid as it may lead to microbial stress shown by disruptions in metabolism, cells' growth or even cells' denaturation [30].

Agitation speed is an important factor in citric acid fermentation as it increases dissolved oxygen concentration in a fermentation medium. Overly high agitation speed results in big mechanical force that may cause damage to cell membranes, changes in microbial morphology, and an increase in aconitase activity, while too low agitation speed will lead to a decrease in citrate synthase activity [31]. Furthermore, [32] examined the effects of agitation speed on the production of citric acid with variations in agitation speed of 300, 400, 500, and 600 rpm. The highest concentration of citric acid was produced at agitation speed of 300 rpm. It was different from another study conducted by [33] who found that maximum concentration of citric acid was produced at high agitation speed of 600 rpm. [32] suggested that the difference between the previously mentioned studies could be caused by several factors: different chemical compositions of the substrates, microbial strains used, and an environmental factor such as dissoved oxygen pressure.

Agitation speed also has impacts on activities of citrate synthase (CS), aconitase (ACH), and isocitrate dehydrogenase (ICDH) [32]. In [32], citrate synthase activities decreased as the agitation speed increased ranging from 300 rpm to 600 rpm. On the other hand, activities of aconitase and isocitrate dehydrogenase increased as the agitation speed increased. Generally, in citric acid production, increasing activities of CS and decreasing activities of ACH and ICDH were found in its cycle.

pH was measured to monitor any decrease in pH level as pH level decrease is an indicator of citric acid yield. Based on the observation performed in this study, pH level decreased in each experiment variation during the fermentation. According to [27], pH of a fermentation medium changes continuously as a result of metabolism activities of the microbes used in fermentation, particularly due to secreted organic acid such as citric acid.

In citric acid production, the pH level of a fermentation medium is a significant factor because *Aspergillus niger* undergoes two phases, namely germination phase and production phase. Spore germination occurs at pH 5 [5]. Germinating spores will absorb nitrogen from trace elements in the form of ammonium nitrate (NH_4NO_3) and release proton (H^+), so the pH decreases. In producing citric acid, with pH ranging from 2.0 ± 0.2, the optimum morphology of *A. niger* will be formed, indicated by small aggregates formation with short filaments. At pH 5, by-products of fermentation such as gluconic acid and oxalic acid will be inhibited. A risk of other microbial contamination can be minimized as well, so it can help citric acid purification. At pH 1.6, the morphology of

A. niger will develop abnormally (*bulbous hyphae)* and citric acid yield will decrease [21].

In their research, [34] demonstrated that pH level affects enzymatic activities in the Krebs cycle. Activities of citrate synthase will be higher from pH 4 until pH 2 as the optimum pH. Activities of isocitrate dehydrogenase and aconitase will decrease at pH from 4 until 2, and pH 4 is the optimum pH. An increase in citric acid yield results from increasing citrate synthase activities and decreasing isocitrate dehydrogenase and aconitase. At pH above 2.5, the main product was gluconic acid. Meanwhile, at pH 2, the main product was citric acid.

As the conclusion of this study, the optimal conditions of citric acid production through submerged fermentation using rice husks as the substrate were as the following: rice husk substrate concentration of 22.42% w/v, sucrose concentration of 10% w/v, methanol concentration of 3% v/v, and agitation speed of 400 rpm. With the optimal conditions mentioned above, the concentration of the citric acid yield was 19.12 g/L at pH 2.34 in 5-day fermentation. In order to enhance citric acid yield, it is necessary to reach the optimum pH, which is 2.0 ± 0.2. Thus, it is recommended that the incubation time be extended or the concentration of NH_4NO_3 be added.

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