Citric Acid Production Optimation from Toba Banana Peel Through Submerged Fermentation by *Aspergillus niger* Using Central Composite Design

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Abstract. Toba Banana (*Musa acuminata* Colla) is a local banana in Toba region, North Sumatera, Indonesia. People only utilize its pulp fruit, then its peels can cause environmental problems. Carbohydrate in banana peel can be utilized as substrate to produce citric acid through fermentation. Citric acid is an organic compound that is widely used in several industries. This research aims to find the optimum condition of Toba banana peel as substrate to produce citric acid through submerged fermentation by *Aspergillus niger*. Parameters observed were substrate concentration 15–30% v/v, sucrose concentration 0–5% w/v, methanol concentration 0–4% v/v, and working volume 100–1000 mL. Sampling was conducted every 24 h during 5 days fermentation to measure its pH and citric acid concentration. The highest citric acid concentration was achieved on day 3 of the fermentation with concentration 15.31 g/L in pH condition 2.12, banana peel concentration of 222.3 g/L (22.23% w/v), sucrose concentration of 26.3 g/L (2.63% w/v), methanol concentration of 21.4 mL/L (2.14% v/v), and working volume 1000 mL. From this result, it can be concluded that Toba banana peel is a potential substrate for producing citric acid by *Aspergillus niger* through submerged fermentation in those optimum conditions.

Keywords: Aspergillus niger · Central composite design · Citric acid · Submerged fermentation · Toba banana peel

1 Introduction

Indonesia is a rich country in abundant tropical fruits. One of the most prominent fruits is banana. Banana is a crop which grows in tropical or subtropical areas and generally known to be rich in vitamins, essential minerals, protein, fiber, lipid, and carbohydrate [1]. In Indonesia, banana grows in most areas, including in Toba region. Toba banana (*Musa acuminata* Colla) has distinguished characteristics. It has seeds and its color stays green despite its being ripe. According to Statistics Indonesia and Directorate General of

A. Lelono et al. (Eds.): ICOLIB 2021, ABSR 27, pp. 216–224, 2023.
https://doi.org/10.2991/978-94-6463-062-6_21

Horticulture Republic Indonesia, the average increase in banana production in Indonesia from 2015–2019 was 0.22%. This is to say that banana waste such as banana peel, bunch, and the others may also increase significantly. Therefore, banana waste utilization is important to prevent future environmental problems.

Banana peel could weigh one third of an unpeeled banana. Its moisture content is about 88.1 g/100 g dwb [2], and banana peel is composed of 60.2% carbohydrate, 8.1% protein, 12.1% fat, and 8.2% fibre [3]. Previous studies have discussed advantages of banana peel aside from being used as animal feed such as antidepressant banana peel candy [4], bioethanol, hydrogen, and methane [5, 6], and citric acid [3].

Citric acid ($C_6H_8O_{7)}$ can be used in foods, beverages, medicine, and cosmetics [7, 8]. It functions to add a sour flavour in various food products. Besides, it can also be used in cleaning products and antioxidant. Moreover, citric acid has been widely used in industries. Twelve percent of its use was in pharmaceutical industry, 70% was in food industry, and 18% was in other industries [9]. That being said, it suggested that citric acid should be produced in large quantities as it has significant economic potential.

Citric acid is produced through a fermentation process using microorganisms. Numerous microorganisms have been used in producing citric acid, namely *Penicillium glaucum, Candida oleophila* [10], *Aspergillus niger* [11], *Aspergillus nidulans* and *Aspergillus awamori* [12], *Hansenula anamola* [13], and *Yarrowia lipolytica* [14]. In this research, *Aspergillus niger* was used to produce citric acid since this microorganism is amongst the commonly used microorganisms to produce citric acid [11, 15, 16]. Furthermore, as the main microorganism used by citric acid producing industries, *Aspergillus niger* can produce larger amount of citric acid and it is also considered economical as it can produce citric acid with low-cost materials [13].

In this study, citric acid was produced through submerged fermentation (SmF). Compared to other methods of fermentation, SmF has more benefits in that it leads to easier product purification, high yield, and time and energy efficiency. Several researchers have investigated citric acid production through SmF [9, 17–19]. In the fermentation process, besides sucrose, methanol can also be used as carbon source. Methanol is useful as it can ferment carbohydrate substrate directly and it can neutralise negative effects of metals in producing citric acid [20]. Methanol addition in stimulating citric acid production may be different depending on the study. For examples, [21] mentioned that the optimum methanol in citric acid production using banana peel was 1% v/v while [22] suggested that using orange peel, the production was 40 mL/kg.

This research was performed to find the optimum conditions of substrate concentration from Toba banana peel, concentration of sucrose and methanol, and working volume in citric acid production through SmF by *Aspergillus niger.*

2 Materials and Methods

2.1 Substrate Preparation

Toba banana trees were widely planted in Toba Region. Toba banana peels were collected from the local fried banana sellers, which means the bananas were in a ripe condition and ready to eat. Banana peels were firstly cleaned and washed by using water. Next, the banana peel was chopped and then dried in an oven at a temperature of 60 °C for 24

h to remove as much moisture as possible. Then, the banana peel was mashed to obtain fine size.

2.2 Cultivation of *Aspergillus niger*

Aspergillus niger was obtained from Microbiology Laboratory, SITH, ITB. *A. niger* was cultivated in Potato Dextrose Agar (PDA) medium and incubated at a temperature of 30 °C for 3 days. The standard curve was generated by making eight variations of *A. niger* inoculum dilutions using NaCl 1% w/v and volume of 10 mL**.** The absorbance was measured using a spectrophotometer UV-Vis at wavelength of 600 nm**.** Then, the sample was centrifuged at a speed of 6000 rpm for 15 min. The pellet was oven-dried at a temperature of 60 °C for 24 h. A linear regression curve was created by plotting the absorbance value versus cell concentration (g/mL). Next, the linear equation was solved. A growth curve was created by taking a sample from inoculated Yeast Peptone Glycerol (YPG) medium with 3 inoculation loops of *A. niger* every two hours**.** The absorbance was then measured again using a spectrophotometer UV-Vis at wavelength of 600 nm**.** Sampling was done until *A. niger*'s growth reached stationary phase**.** The absorbance value was converted to cell concentration (g/mL) using linear equation generated from the standard curve. Then, a graph of the growth curve of the cell concentration versus time was created**.** Based on the growth curve, the inoculum age in exponential phase and to be used in the fermentation was 36 h, or with absorbance 0.39 at wavelengths of 600 nm and concentration of 23.2 g/mL.

2.3 Standard Curve of Citric Acid

The citric acid standard curve was generated by making eight different citric acid concentrations from stock solution concentration of 0.1 mg/mL. The absorbance was measured by using a spectrophotometer UV-Vis at wavelength of 407 nm. Then, a linear regression curve of absorbance and citric acid (mg/mL) was generated and the linear equation was solved.

2.4 Fermentation

The fermentation medium was composed of NH_4NO_3 0.25% w/v, KH_2PO_4 0.1% w/v, and $MgSO_4$ 0.025% w/v of working volume. Five percent of working volume was taken from the fermentation medium, inoculated with 3 inoculation loops of *Aspergillus niger,* then incubated at 30 °C for 36 h. As much as 5% of the inoculum of the working volume was inserted into the fermentation medium. The fermentation was performed in a medium with a magnetic stirrer at a temperature of 30 °C for 5 days. During the fermentation, sampling was carried out every 24 h.

2.5 pH and Citric Acid Concentration Determination

Every 24 h, 6 mL of sample was taken with three times repetitions. The sample was centrifuged at a speed of 6000 rpm for 15 min. The supernatant was separated into a

new Falcon tube. pH level of 3 mL and then measured using a pH meter. The citric acid concentration was determined by adding 1.3 mL pyridine into the 3 mL supernatant to be homogenized. Next, 5.7 mL acetic anhydride was added to be homogenized using vortex. The solution sample was then incubated at a temperature of 32 °C for 30 min until its color turned light yellow. The solution absorbance was measured using spectrophotometer UV-Vis at wavelength of 407 nm. The absorbance value was converted into citric acid concentration (mg/mL) using standard linear regression curve of citric acid.

2.6 Experiment Variations and Analysis

Experiment variations on concentration parameter of Toba banana peel substrate, sucrose and methanol concentration, and working volume were determined by applying Central Composite Design-Response Surface Methodology as statistical approach, which the variations can be seen in Table 1. Data on citric acid concentration resulted from every parameter was analyzed using Minitab to achieve optimum parameter conditions and maximum citric acid concentration.

3 Results and Discussion

Based on the data analysis shown in Table 2, it can be seen that on day 3 of fermentation, it reached the optimum production of citric acid concentration of 15.31 g/L, pH 2.12, banana peel concentration of 222.3 g/L, sucrose concentration of 26.3 g/L, methanol concentration of 21.4 mL/L, and optimum working volume of 1000 mL.

In this study, citric acid fermentation was performed in 5 days. On each day were pH level and citric acid concentration measured. Maximum citric acid concentration of 15.31 g/L was recorded on day 3. After that day, the more fermentation duration, the lower citric acid concentration. It resulted from decreasing carbon source both from the substrate and the sucrose, decreasing nitrogen source, and produced metabolic inhibitor in the fermentation medium [23]. The carbon source from polysaccharide in the banana peel substrate was hydrolysed by hydrolysis enzyme which was effectively produced by *A. niger* at pH 1.8–2.2. At that pH range, *A. niger* morphology was in the form of small aggregates with short filaments, which was the optimum *A. niger* morphology in citric acid fermentation [12]. In Table 2, it could be seen that the average of the medium pH level on day 1 of the fermentation was 4.97 and therefore the medium pH level at the beginning (day 0) was above 5. [12] said that pH above 5 at the beginning of a fermentation process stimulated germination and spore formation. Increased number of produced spores led to greater absorption of ammonia which was a nitrogen source and proton release. These conditions resulted in lower pH level and therefore higher citric acid production. With decreasing pH level at the range of 2, production of by-product of citric acid and contamination caused by other microorganisms could be inhibited. Thus, citric acid production could be enhanced.

Substrate is the nutrition source for microorganisms to metabolise and to produce citric acid. In this study, banana peel substrate was used in order to utilise organic waste. Numerous researches discussed on using organic waste from fruits' peels as fermentation substrate such as orange peel, lemon peel, mango peel, and pineapple peel which yielded

Table 1. Experiment variations of fermentation conditions

Run	Sub-strate (%w/v)	Sucrose (%w/v)	Methanol (%v/v)	Working volume (mL)
1	15	0	0	100
2	30	0	0	100
3	15	5	0	100
4	30	5	0	100
5	15	0	4	100
6	30	0	4	100
7	15	5	4	100
8	30	5	4	100
9	15	0	0	1000
10	30	0	0	1000
11	15	5	0	1000
12	30	5	0	1000
13	15	0	4	1000
14	30	0	4	1000
15	15	5	4	1000
16	30	5	4	1000
17	22.5	2.5	2	550
18	22.5	2.5	2	550

citric acid with concentration between 5–11.34 g/L [24, 25]. While functioning as carbon source, substrate also becomes one of nitrogen and phosphate sources. Nitrogen and phosphate deficiency in the substrate required during fermentation could be addressed by adding nitrogen source such as potassium nitrate or ammonium nitrate, and dipotassium phosphate or potassium dihydrogen phosphate [26]. In this research, ammonium nitrate 0.25% w/v and potassium dihydrogen phosphate 0.1% w/v were added into the substrate as performed in [24, 27]. Adding ammonium nitrate inhibited the growth of *A. niger* vegetation and the concentration of 0.25% w/v limited the accumulation of oxalic acid which could lessen citric acid production [26]. Meanwhile, phosphate addition should be limited as it could induce biomass growth, which can affect the production of citric acid [12].

Carbon is the main component which directly affects the fermentation process to produce citric acid. Compared to polysaccharide, carbon sources in the form of monosaccharide and disaccharide are more easily metabolized by microorganisms to yield larger quantities of citric acid. Nonetheless, using only monosaccharide and disaccharide in producing citric acid at industrial scale is not efficient. In that case, additional carbon source is required in the form of polysaccharide from organic waste. A few commonly

Table 2. Optimal concentration of banana peel substrate, sucrose, and methanol during 5-day submerged fermentation by *Aspergillus niger*

Citric acid concen-tration (g/L)	Optimal concentration			Day	pH
	Substrate (g/L)	Sucrose (g/L)	Methanol (mL/L)		
6.16	222.3	26.3	21.4	1	4.97
10.59				2	3.19
15.31				3	2.12
14.72				4	2.57
10.27				5	3.23

used carbon sources in the form of monosaccharide and disaccharide in citric acid production are glucose, maltose, fructose, and sucrose [25, 27, 28]. In the aforementioned studies, additional carbon sources used were pineapple peel, orange peel, and banana peel. It was also suggested that the use of sucrose as carbon source produced citric acid with maximum concentration since it is composed of two monosaccharides. Optimum sucrose concentration may be different in studies about citric acid production depending on the type and concentration of substrate used as carbon source, and other fermentation conditions. In this study, maximum citric acid concentration of 15.31 g/L was yielded using optimum sucrose concentration of 26.3 g/L and optimum banana peel substrate concentration of 222.3 g/L.

Besides monosaccharide and disaccharide, alcohol is one of the main factors affecting citric acid production. Based on several studies on producing citric acid through fermentation, the types of alcohol used in the fermentation were methanol, ethanol, and isopropyl alcohol whose concentration was between 1–4%v/v [18, 28, 29]. Those studies showed a tendency that using the same concentration of methanol resulted in higher concentration of citric acid compared to using ethanol or isopropyl alcohol. Ingram & Buttke (1984) and Vandenberghe et al. (1999) as cited in [20] argued that methanol could increase citric acid production as it reduced the effect of metal ion inhibition. Adding methanol (molecular weight of 32.04 g/mol) could enhance cell wall permeability and cause metal ions to permeate the cells without breaking the cell membrane like what happens in alcohol use with higher molecular weight, but because phospholipid structure and cell wall protein are disrupted [20, 30]. Low-concentration metal ions play a role in promoting organic compounds anabolism by *A. niger,* including as a stimulator in the synthesis of citric acid, as a catabolism inhibitor, and as an enzyme and cofactor catalyst [12, 31]. In this research, the use of optimum methanol concentration of 21.4 mL/L or 2.14% v/v could produce citric acid with maximum concentration of 15.31 g/L. In the study conducted by [20] on comparing an addition of methanol with concentration of 1–5% v/v into a fermentation medium containing molasses and corn cobs as carbon sources to a control group with no addition of molasses and corn cobs. The result showed that maximum concentration of citric acid produced was 16.3 mg/mL in the medium with molasses and methanol 2% v/v.

As the conclusion, the optimum conditions to produce citric acid with maximum concentration of 15.31 g/L through SmF by *Aspergillus niger* were as follows: Optimum concentration of Toba banana peel substrate was 222.3 g/L; optimum sucrose concentration was 26.3 g/L; optimum methanol concentration was 21.4 ml/L; and optimum working volume was 1000 mL.

Acknowledgments. This research was financially supported by LPPM Institut Teknologi Del (No. 012.2/ITDel/LPPM/ Penelitian/VII/2020), Toba, North Sumatera, Indonesia.

Authors' Contributions. MMM designed the research, analyzed the data dan wrote the manuscript, AM designed the research and supervised all the process, HNH and FBAF conducted the research process and collected the data. All authors read and approved the final manuscript.

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