The production of citric-acid from Bilimbi (Averrhoa bilimbi) fruits through the fermentation process using Aspergillus niger

A Y A Aritonang, E Julianti* and H Sinaga

Department of Food Science, Faculty of Agriculture, Universitas Sumatera Utara, Medan, Indonesia.

E-mail: *elisal@usu.ac.id

Abstract. The aim of this study was to evaluate the quality of citric acid products made from bilimbi fruits by fermentation method using Aspergillus niger as inoculum at various levels of concentration (3%, 6%, and 9%) and fermentation time (1, 3, 5, and 7 days). The citric acid obtained was then analysed their quality including yield, total sugar content, and citric acid content. There is significantly different of citric acid content as affected by the interaction of inoculum concentration and fermentation period, but no significantly different effect on yield and total sugar of fermentation product. The best fermentation condition for citric acid production from bilimbi fruit juice was 6% inoculum concentration with 5 days fermentation. At this condition the citric acid concentration was 3.33%.

1. Introduction

Bilimbi (Averrhoa bilimbi L.) is a plant from Indonesia and mainland Malaya which is widely found because of its easy planting and does not require special care [1]. Several studies reported that, bilimbi contains active compound such as saponins, glucosides, tannins, flavonoids, citric acid, formic acid, contain pectin compounds, flavonoids, and vitamin C, minerals, calcium oxalate and potassium [2].

Citric acid with the molecular formula $C_6H_8O_7$ (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a weak organic acid that can be obtained from citrus plants, but with the development of science and technology applied, it can be produced from other plant sources or synthesis (microbial fermentation or chemical synthesis). This acid has relative high solubility, is safe for consumption, and produces sour taste. Citric acid can be used as a preservative to prevent deterioration of colour and aroma, inhibit oxidation, convert sucrose into glucose, give dark colour on confectionery products and to adjust the acidity of jam and jelly. Nowadays, citric acid was produced industrially through microbial fermentation by Aspergillus niger, Aspergillus wentii, Aspergillus awamori, or Aspergillus nidulans from various plants or raw materials that were available and cheap. The raw materials come from corn cobs, rapeseed oil, kiwi fruit skins, orange, apples or beer manufacturing waste [3].

The basic principle of fermentation is to activate certain microbial activities with the aim of changing the properties of the material to produce something useful. During the fermentation process the number of microbes is doubled and their metabolism in these materials is activated to a certain extent [4]. Moreover, in the process, moulds need a substrate which is the most important nutrient for moulds. When growing, A. niger mould will be directly related to the nutrients in the substrate. The basic molecules that are above the hyphae can move directly while the more complex molecules such as protein, starch, cellulose, and proteins must be broken down or separated before being absorbed into the...
cell to produce some extracellular enzymes. The organic matter in the substrate is used by the Aspergillus niger fungus for transport activities, maintenance of cell structures and cell movement [5].

Bilimbi juice fermentation using Aspergillus niger plays an important role to produce citric acid. The quality of citric acid product from bilimbi through fermentation using inoculum of A. niger at various levels of concentration and fermentation time were evaluated in this study. These findings are expected to provide information about bilimbi as a potential source of citric acid.

2. Materials and methods

Bilimbi fruits used in this study was obtained from Binjai City, North Sumatera, potatoes, potato dextrose agar. The cultures stock of Aspergillus niger obtained from the microbiology laboratory in Faculty of Pharmacy, Universitas Sumatera Utara. The reagents used in in the analysis were distilled water, sucrose, magnesium sulphate, potassium dihydrogen phosphate, ammonium nitrate, zinc sulphate, ferroammonium sulphate, oxalic acid, phenolphthalein, NaOH, and HCl.

2.1 Preparation of A. niger maintenance

The culture stock of A. niger was used for producing of citric acid by fermentation process. The culture of A. niger was grown in 1.5% sterilized potato dextrose agar (PDA), and incubated at 28°C for 7 days until spore of A. niger spores formed and it will be used as inoculum in citric acid fermentation [6].

2.2. Preparation of mineral stock solution

Mineral stock solution was prepared separately consists of 3g/100mL MgSO\(_4\) 7H\(_2\)O, 2g/100 mL KH\(_2\)PO\(_4\), 4 g/100 mL NH\(_4\)NO\(_3\), 10 mg/100 mL FeSO\(_4\) 7H\(_2\)O, and 25 mg/100 mL ZnSO\(_4\) 7H\(_2\)O. Each mineral; stock solution was sterilized at 121 °C for 15 min in an autoclave, cooled and stored in refrigerator [7].

2.3. Preparation of growth media for A. niger

Growth media was prepared according to Kareem [7] methods. 40 g sucrose was dissolved with aquadest in a 1L volumetric flask until the mark, pH was adjusted to be 5.8 using HCl 1M, and then sterilized. The mineral stock was added aseptically to the media growth as much as 1.5 mL MgSO\(_4\) 7H\(_2\)O; 1.5 mL KH\(_2\)PO\(_4\); 1.5 mL NH\(_4\)NO\(_3\); 0.1 mL Fe\(_2\)SO\(_4\) 7H\(_2\)O; and 0.1 mL ZnSO\(_4\) 7H\(_2\)O.

2.4. Preparation of vegetative inoculum

Small amount of spore from the slant PDA was suspended in 10 mL 0.05% Tween 80. 2% spore suspension was transferred aseptically to the sterilized 250 mL Erlenmeyer flask and 150 mL growth medium was added and suspended. The suspension then was incubated in shaker incubator (150 rpm) at 30 °C for 4 days [7].

2.5. Preparation of production medium

500g bilimbi fruits was blended in 1 L distilled water by using blender and filtered. The pH of bilimbi juice was adjusted to 3.5 by using NaOH 1 M. The media then was sterilized, and the solution stock of Fe\(_2\)SO\(_4\) 7H\(_2\)O (1 mL) and ZnSO\(_4\) (1 mL) were added [8].

2.6. Citric acid production

The production of citric acid was done in a fermenter such as 250 mL Erlenmeyer flasks. 150 mL production media was put into fermenter, and vegetative inoculum of A. niger was added aseptically at different concentration (K\(_1\) = 3% v/v, K\(_2\) = 6% v/v, K\(_3\) = 9% v/v), and fermented at 30 °C in incubator shaker (150 rpm) with different fermentation time namely 1 days (F\(_1\)), 3 days (F\(_2\)), 5 days (F\(_3\)) and 7 days (F\(_4\)). Fermentation products containing citric acid then was sterilized at 121 °C for 15 min, and then was analysed to their yield (the weight of citric acid product from fermentation is divided by the weight of the bilimbi fruits juice used), total sugar [9], titratable acidity [10], and citric acid content [11].
2.7. Data analysis
The design used in this research was a factorial completely randomized design with two factors, namely the concentration of A. niger inoculum as first factor (3%, 6%, and 9%), and fermentation periods as second factor (1 day, 3 days, 5 days, and 7 days). Each combination of treatment was done in 3 replicates. The observation data was analysed by analysis of variance, and if the treatment gave significantly effect, the further testing will be carried out by using Least Significant Range test.

3. Results and discussion

3.1. Yield
Analysis from data shows the inoculum concentration has a significant (p<0.05) effect, but fermentation time and the interaction between inoculum concentration and fermentation time did not gave the significantly different (p > 0.05) on the citric acid yield (Table 1).

Table 1. Citric acid yield from bilimbi fruit juice as affected by A. niger concentration and fermentation time.

| Inoculum concentration | Fermentation Time | Effect of Inoculum concentration effect |
|------------------------|------------------|----------------------------------------|
|                        | F1= 1 day        | F2 = 3 days | F3 = 5 days | F4 = 7 days |
| K1 = 3%                | 28.47±0.23       | 28.33±0.31 | 29.67±0.70 | 28.40±0.69 |
| K2 = 6%                | 32.40±1.74       | 31.07±0.92 | 32.80±0.31 | 30.47±0.95 |
| K3 = 9%                | 33.33±1.03       | 32.87±0.58 | 32.67±0.35 | 31.33±0.37 |
| Effect of Fermentation Time | 31.40±2.58 | 30.76±2.29 | 31.58±1.66 | 30.07±1.57 |

The different superscript letters that follow the value in the same column are significantly different (p<0.05) by LSR test.

Table 1 shows the increasing concentration of A. niger inoculum added to the substrate media can produce the higher citric acid yield. The addition of an inoculum on the substrate which can improve the performance of spores A. niger so as maximize citric acid yield. The concentration of inoculum as much as 1.0% is sufficient in production of citric acid. The increase in the number of inoculated spores was able to increase the citric acid yield. This occurs due to the addition of an inoculum on the substrate which can improve the performance of Aspergillus niger spores so as maximize citric acid yield. Approximately 1.0% of the vegetative inoculum is sufficient for optimal citric acid production. Increasing the number of inoculated spores can increase the yield of citric acid obtained [3].

3.2. Total sugar content
Analysis of the data showing that the concentration of inoculum, fermentation period, and the interaction between concentration of inoculum and fermentation period gave an effect with significantly different (p<0.05) on the total sugar citric acid (Table 2).

Based on Table 2 it can be seen that the total sugar produced from citric acid increases with the number of inoculums added. The higher the inoculum concentration added, the greater the number of fungi A. niger that can convert carbohydrates into glucose. In this case, it is due to the presence of carbohydrates compounds which are a source of carbon that convert glucose for development and growth [12]. The amount of inoculum added to the production medium resulted in a growth population of compacted A. niger so that it produced sugar faster. The longer the fermentation is carried out, the total sugar will decrease. Therefore, the highest total sugar content was obtained during fermentation for only 1 day. This is because the longer the fermentation take place, the mould continues to develop and grow following its growth phase. The increase in the number of moulds causes more sugar to be used for growth and metabolism of moulds so that glucose decreases [13].
Table 2. Total sugar content from bilimbi fruit juice as affected by *A. niger* concentration and fermentation time.

| Inoculum concentration | Fermentation Time | Effect of Inoculum concentration effect |
|-------------------------|-------------------|----------------------------------------|
|                         | F1 = 1 day        | F2 = 3 days                            | F3 = 5 days | F4 = 7 days |
| K1 = 3%                 | 0.77±0.03         | 0.60±0.03                              | 0.49±0.00   | 0.37±0.00   | 0.56±0.17b |
| K2 = 6%                 | 0.81±0.00         | 0.66±0.04                              | 0.53±0.03   | 0.40±0.02   | 0.59±0.18b |
| K3 = 9%                 | 0.91±0.03         | 0.78±0.00                              | 0.58±0.00   | 0.45±0.00   | 0.67±0.20b |
| Effect of Fermentation Time | 0.77±0.07a | 0.67±0.00                              | 0.55±0.05a  | 0.42±0.04a  |

The different superscript letters that follow the value in the same column are significantly different (p<0.05) by LSR test.

3.3. Titratable acidity

Analysis of the data show that the inoculum concentration, fermentation time, and the interaction between inoculum concentration and fermentation time gave an effect with significantly different (p<0.05) on titratable acidity in Table 3.

Table 3. Titratable acidity from bilimbi fruit juice as affected by *A. niger* concentration and fermentation time.

| Inoculum concentration | Fermentation Time | Effect of Inoculum concentration effect |
|-------------------------|-------------------|----------------------------------------|
|                         | F1 = 1 day        | F2 = 3 days                            | F3 = 5 days | F4 = 7 days |
| K1 = 3%                 | 3.25±0.07         | 3.56±0.08                              | 4.55±0.19   | 5.05±0.28   | 4.10±0.84c |
| K2 = 6%                 | 3.39±0.19         | 4.01±0.21                              | 4.8±0.20    | 5.23±0.13   | 4.36±0.82b |
| K3 = 9%                 | 3.87±0.20         | 4.29±0.16                              | 4.83±0.32   | 5.42±0.27   | 4.60±0.67a |
| Effect of Fermentation Time | 3.50±0.33d | 3.95±0.37c                             | 4.73±0.15b  | 5.23±0.19a  |

The different superscript letters that follow the value in the same column are significantly different (p<0.05) by LSR test.

Table 3 shows that the resulting inoculum concentration affects titratable acidity of products. The highest inoculum concentrate produced less total acidity. This is because the ability of the mould to produce acid is influenced by the amount of inoculum added. So, when addition needs to be considered so that the mould can produce citric acid to the maximum [13]. Mould mycelia are too dense in producing citric acid which can interfere with mould growth and development [14]. Titratable acidity increased with fermentation time which was up to 5 days because the mould could multiply their cells rapidly and the availability of nutrients in the media. It meant that acid production was optimal at 5 days. This is because the mould has entered the stationary phase (permanent growth) and continues to divide and is resistant to the environment even though the available nutrients have been reduced and the presence of toxic compounds that result in cell growth. Titratable acidity decreased at 7 days fermentation and through a phase of death due to unavailability of nutrients and nitrogen levels in the media so that mould cannot survive [15].

3.4. Citric acid content

The interaction effect between inoculum concentration and fermentation time on the citric acid content from fermentation product of bilimbi juice by *A. niger* can be seen in Table 4. The highest levels of citric acid were obtained at an inoculum concentration of 6% and fermentation time of 5 days, namely 3.33%. This is because the ability of the mould to produce citric acid is influenced by the amount of
inoculum added. So that in addition it needs to be considered so that the mould can produce citric acid maximally [8]. Fungal mycelia are too dense to produce citric acid which can interfere with mould growth and development [14].

In the first day fermentation time, the production of bilimbi fruit citric acid levels was still low because *A. niger* experienced a lag phase that adapted to the environment of the fermentation medium and cell division had not started because the enzyme had not been synthesized. Then the F2 treatment (3 days), an increase in citric acid levels because the fungi can divide quickly and the availability of nutrients in the media. Furthermore, in F3 treatment (5 days), citric acid production was optimal. This is because the mould has entered the stationary phase (permanent growth) and continues to divide and is resistant to the environment even though the available nutrients have been reduced and the presence of toxic compounds which results in cell growth. In F4 treatment (7 days), citric acid levels decreased and experienced a death phase due to the unavailability of nutrients and nitrogen levels in the media so that the mould could not survive [15]. The production of citric acid from brem solid waste using the fermentation static cultivation method for 1 day obtained the highest citric acid of 3.84 g / l [16].

**Table 4.** Citric acid content from bilimbi fruit juice as affected by *A. niger* concentration and fermentation time. 

| Inoculum concentration | Fermentation Time | Effect of Inoculum concentration effect |
|------------------------|-------------------|----------------------------------------|
|                        | F1 = 1 day        | F2 = 3 days                            | F3 = 5 days                            | F4 = 7 days                            |
| K1 = 3%                | 2.86±0.01         | 2.71±0.02                              | 2.65±0.03                              | 3.00±0.02                              |
| K2 = 6%                | 2.81±0.01         | 2.62±0.02                              | 3.33±0.04                              | 3.02±0.04                              |
| K3 = 9%                | 2.81±0.03         | 3.12±0.04                              | 2.97±0.02                              | 2.75±0.05                              |

Effect of Fermentation Time: 2.83±0.03 2.82±0.27 2.98±0.34 2.92±0.15

The different superscript letters that follow the value in the same column are significantly different (p<0.05) by LSR test

During the production of citric acid (idiophase), the activity of the enzyme citrate synthase will increase up to 10 times. Furthermore, the enzyme activity breaks down citric acid which catabolizes citric acid such as aconitase and isocitric dehydrogenase decreased compared to the activity during tropophase. Then, the TCA acid cycle stops when citric acid is taken and the synthase enzyme is unable to work alone to keep the TCA cycle going [17].

### 4. Conclusions

In this present research, Aspergillus niger can be used to support the production of citric acid from bilimbi fruits juice through fermentation process. The optimal condition for the maximum production of citric acid reached at 6% inoculum of *A. niger* and 5 days fermentation time. At this condition the yield of fermentation product obtained was 32.80% with citric acid content of 3.33%.

### References

[1] Oktaviana D 2012 Combination of Maltodextrin and Heating Temperature on Quality of Instant Starfruit Powder Drink (Avverhoa bilimbi Linn) Thesis (Yogyakarta: Universitas Atma Jaya) p 37

[2] Masruhen 2010 The effect of infusion of star fruit (*Avverhoa bilimbi* L.) on blood cholesterol levels of rats *J Pharm* 1 3

[3] Max B, Salgado J M, Rodriguez N, Cortes S, Converti A and Dominguez J M 2010 Biotechnological production of citric acid *Brazil J of Micro* 4 865

[4] Assegaf F 2009 *Prospek Produksi Bioetanol Pisang (Musa paradisiaca L) Menggunakan Metode Hidrolisis Asam dan Enzimatis* [Production Prospects of Bioethanol Bananas (*Musa paradisiaca* L) Using Acid and Enzymatic Hydrolysis]
Using Acid and Enzymatic Hydrolisis Methods] Thesis (Jawa Tengah: Universitas Jenderal Soedirman) p 65

[5] Hardjo S, Indrasati N S and Bantacut T 1998 Biokonversi : Pemanfaatan Limbah Industri Pertanian [Bioconversion: Utilization of Agricultural Industry Waste] (Bogor: IPB-Press Bogor) 54

[6] Carolina A, Sidik A, Maksum I P, Rachman S D, Safari A and Ishmuyana S 2015 Fermentasi Biak Rendam Molases dengan Aspegillus niger untuk Produksi Asam Sitrat [Submerged Fermentation of Molases by Aspergillus niger for Citric Acid Production] Chimica et Natura 3 1 pp 25-9

[7] Kareem S O, Akpan I and Alebiowu O O 2010 Production of citric acid by Aspegillus niger using pineapple waste Malaysian Journal of Microbiology 6 2 pp 161-5

[8] Abbas N, Safdar W, Ali S, Choudry S and Elahi S 2016 Citric acid production from Aspergillus niger using mango (Mangifera indica L.) and sweet orange (Citrus sinensis) peels as substrate Int J of Scien & Eng Resch 2 568

[9] Apriyantono A, Fardiaz D, Puspitrasari N L, Sedarnawati and Budiyanto S 1989 Analisis Pangan [Food Analysis] (Bogor: PAU Pangan dan Gizi IPB Press)

[10] Ranganna S 1986 Hand Book of Analysis and Quality Control for Fruit and Vegetable Products 2nd ed (New Delhi: Tata McGrow-Hill Publishing Co Ltd) pp 8-229

[11] Marier J R and Boulet M 1958 Direct determination of citric acid in milk an improved pyridine-acetic anhydride method J Dairy Sci 41 pp 1683-92

[12] Betiku E 2013 Optimization of sweet potato starch hydrolzate production and its potential utilization as substrate for citric acid promotions Bri Biot Jour. 2 172

[13] Jayabalan R, Malbasa R V, Loncar E S, Vitas J S and Sthiskumar M 2014 A review on kombucha tea microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus Compre Rev in Food Science and food Safety 4 542

[14] Mattey M and Allan A 1990 Glycogen accumulation in Aspergillus niger Trans of Biochem Soc 1 1021

[15] Bekir S, Kariptas E and Ciftci H 2009 Effects of fermentation conditions on citric acid production from beet molasses by Aspergillus niger Asia J Chem 4 3215

[16] Kusuma G A, Antara N S and Suwariani N P 2019 Fermentation of citric acid production using Aspergillus niger ATCC 16404 with industrial solid waste liquid hydrolysate substrate brem J of Agro Eng and Manag 4 620

[17] Haryani,K 2011 Study of growth kinetics Aspergillus niger on citric acid fermentation from pineapple peel in reactor an external loop air-lift J of Mom 1 50