Comparison of Addition Mold and Yeast Inoculants on the Production of Citric Acid in Liquid and Solid Media from Sorghum

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Abstract. The aim of the study was to determine the production of citric acid in liquid and solid media made from the juice and bagasse of sorghum plant. Sorghum has a great potential to be utilized as fermentation substrate for production of citric acid. The production of citric acid by fermentation is influenced by several factors including the type of media and used microorganisms. Fermentation to produce citric acid can be done by using mold as well as yeast. In this study, the mold isolates were Beauveria bassiana and Paenomyces lilacinus, while the yeast isolates were Candida utilis and Candida lessepsii. The fungal fermentations were conducted in two phases of medium, liquid and solid. Three formulas were prepared for liquid fermentation, namely sorghum juice only, sorghum juice supplemented with NH₄NO₃ and micronutrients, and sorghum juice supplemented with asparagine and micronutrients. Two formulas were prepared for solid fermentation, sulfuric acid hydrolyzed sorghum bagasse and non-hydrolyzed sorghum bagasse. The results showed that citric acid yield in solid fermentation is lower than liquid fermentation. For all formulas, the citric acid formation through liquid fermentation was higher in yeast than in molds. On the other hand, for solid fermentation, citric acid production was higher in hydrolyzed media than non-hydrolyzed media, except for yeast Candida lessepsii.

1. Introduction
Citric acid has been widely utilized in various industrial purposes. Approximately 60% of total citric acid products were used by food industry, while pharmaceutical industry put to use 30% of them, and the rest were handled as preservatives or flavoring agents [1]. Naturally, citric acid can be found in fruits such as orange, pineapple, and pear. Fungal fermentation production of citric acid was firstly investigated in 1923 as a by-product of calcium oxalate produced by a culture of Penicillium glaucum from fermenting sugars [2].

Sorghum is a one of crops that have high adaptability in marginal land particularly under limitation of water availability. This beneficial feature makes sorghum a good alternative crop to meet global needs for foods, renewable energy source, as well as various industrial demands. Varying among cultivars, averagely about 17.8 to 40.3% on dry weight basis of sorghum stalk contains total sugars that available for sugar fermentation [3]. Those sugars mainly consist of sucrose, glucose, and fructose.

Generally, fungal fermentation process was run on two steps, starting with mycelial growth and then followed by fermentation to produce the products. Both steps utilized carbohydrates. Various factors influence the production of citric acid in fermentation broth, with the major factor relies on the composition of media such as carbon source, nitrogen source, phosphorus source, as well as the utilized
Microorganism [4]. Molds such as *Aspergillus niger*, *Trichoderma viridae*, *Paecilomyses lilacinus*, and *Beauveria bassiana* have been widely used in the formation of citric acid. Commercially, production of citric acid nowadays is mostly produced by microbial fermentation using *Aspergillus niger* [5]. In deep investigation on metabolic pathway, *A. niger* can produce abundance citrate via an active glycolytic pathway. Beside molds, many yeasts were also indicated to have ability to produce citric acid. Other than *Saccharomyces cerevisiae*, *Candida* genera can also be considered for citric acid fermentation due to its beneficial properties such as high growth rate and low doubling time [6].

The demand of citric acid is rapidly increased every year; thus, the optimization of its production is required. The utilization and selection of microbes for fermentation process among other factors that influence the production of citric acid need to be investigated thoroughly. Furthermore, the production of citric acid is caused by the cumulative result of various nutritional conditions in the medium. Therefore, the aim of this study was to understand the effect of different microorganism group, yeast and mold, toward the formation of citric acid under fermentation process by utilized sorghum stalks as medium.

2. Materials and Methods

2.1. Microorganisms

All microorganisms, molds and yeasts, were obtained from Indonesian Culture Collection (InaCC). The mold isolates were *Beauveria bassiana*, *Paecilomyces lilacinus*. The yeast isolates were *Candida utilis* and *Candida leesepsi*. All isolates tested were first grown in Potato Dextrose Agar (PDA) medium at 30°C for 72 hours.

2.2. Fermentation Media

The fungal fermentations were conducted in two phases of medium, liquid and solid. Both medium originated from sorghum stalks. The liquid media, mainly consist of sorghum juice, extracted from the sorghum stalk, while the solid fermentation media utilized its bagasse.

2.3. Preparation of Liquid Media for Fermentation

The sorghum juice was obtained by squishing the sorghum stalks. Three formulas were prepared for liquid fermentation. Formula 1 contained 500 mL of fresh sorghum juice only. Formula 2 contained 500 mL of fresh sorghum juice with supplementation of NH\(_4\)NO\(_3\) 1.5 gram, KH\(_2\)PO\(_4\) 0.5 gram, MgSO\(_4\) \(\cdot\) 7H\(_2\)O 0.2 gram, FeSO\(_4\) 7H\(_2\)O 0.05 gram, and KCl 0.2 gram. Preparation of formula 3 was similar to formula 2, with substitution of NH\(_4\)NO\(_3\) with asparagine 1 gram. All liquid formulas were homogenized then wet sterilized in autoclave at 121°C for 15 minutes. As 20 mL of the broth then transferred to 50 mL Falcon tubes.

2.4. Preparation of Solid Media for Fermentation

Two formulas were prepared for solid fermentation, using sorghum bagasse as main ingredients. The bagasse was hydrolyzed using H\(_2\)SO\(_4\) as Formula 1. For mold fermentation, about 5 grams of the bagasse were hydrolyzed with H\(_2\)SO\(_4\) 5%, while H\(_2\)SO\(_4\) 0.005% was added to 10 grams of bagasse for yeast fermentation. The non-hydrolyzed bagasse was treated as Formula 2. All solid formulas were wet sterilized in autoclave at 121°C for 15 minutes.

2.5. Inoculation of mold and yeast into liquid and solid media

The molds were transferred from surface of PDA to liquid and solid media by using tip micropipette. The yeasts were firstly diluted into sterile water (OD 2.0), then 1 mL of the broth was transferred to liquid and solid media. All media were kept at 30°C. The incubation time for liquid media was 5 x 24 hours, and 7 x 24 hours for solid media. The experiment was conducted with three repetitions for each concentration.

2.6. Measurement of level of acidity
The level of acidity was measured by using pH meter (WA-2015 Lutron) directly to liquid fermented broth, but for the solid media, 1 gram of bagasse was mixed with 5 mL of sterile water prior to measurement.

2.7. Quantitative determination of Reducing Sugars
The consumed reducing sugars after incubation was estimated using dinitrosalicylic acid (DNS) method. The reagent was prepared by mixing 0.5 gram of dinitrosalicylic acid and 1 gram of NaOH pellet into 50 mL of water, then boiled for 10 minutes. The glucose solution in the range concentration of 0 – 1000 ppm was prepared as standard solutions.

The sample of solid media was first mixed vigorously with water (1:9). This diluted sample along with liquid fermentation broth were centrifuged with velocity of 7000 rpm at 4°C for 10 minutes. The supernatants and standards solution were taken as much as 1 mL then mixed with 0.5 mL of the DNS reagent in test tube. The mixture was bathed in boiled water for 10 minutes. The color developed was measured at 530 nm with a spectrophotometer UV-Vis (JK-VS-721N, JKI, China). Concentration of reducing sugars was calculated using a standard curve prepared with standard glucose after correction with the absorbance of uninoculated control at 540 nm.

2.8. High Performance Liquid Chromatography (HPLC) quantification of citric acid
The citric acid concentration after fermentation period was measured using HPLC. The analysis was performed following these conditions: reverse phased C18 Cosmosil (15 cm x 4.6 mm) column as stationary phase and methanol : NaH2PO4, H2O 50 mM pH 2.4 (11: 89) as mobile phase with flow rate 1 mL/minutes. The analyte was detected by using UV-Vis detector at 215 nm. The citric acid solution in the range concentration of 0 – 1000 ppm was prepared as standard solutions. The sample of solid media was first mixed vigorously with water (1:9). This diluted sample along with liquid fermentation broth were centrifuged with velocity of 7000 rpm at 4°C for 10 minutes. Both the supernatant samples and standard solutions were filtered through 0.45 µM Whatman paper. As much 20 µL of samples and standards was injected to set up instrument. Citric acid concentration of sample was calculated using a standard curve prepared with standard citric acid.

3. Results and Discussion
3.1. Level of acidity
After incubation, the level of acidity of each formula both in liquid and solid media were measured. Table 1 shows the pH of liquid fermented media by the molds and yeasts after incubation. In general, the level of acidity of liquid media for each treatment was drop after fermentation, with mostly below 1 point. Averagely, liquid fermentation with yeasts decreased more pH than using molds. Fermentation with yeast Candida lessepsii resulted the most decreasing pH for each formula, while Beauveria bassiana was least descended the pH of media. Uninoculated media were treated as control for each formula.

Solid fermentation with molds and yeasts was also resulted the changing of media. The sorghum bagasse as the media was divided into two treatments, with hydrolysis of sulfuric acid and without additional of the acid. The concentration of acid added was also different. The media for inoculation with molds was hydrolyzed with high concentration of H2SO4 namely 5%, while 0.005% only was added to yeast fermentation. The initial acidity influences the ability of fungi to grow and survive. Yeasts have ability to grow in a pH range of 4 to 4.5 and molds can grow from pH 2 to 8.5, but prefer acidic condition [7], hence the concentration of acid for hydrolysis of bagasse is different for mold and yeast fermentation. Acid hydrolysis was conducted before fermentation to digest and release total sugars that will be used as main available carbon sources for fungal fermentation [8].

| Inoculants | Species          | pH        |
|------------|------------------|-----------|
|            | Formula 1 | Formula 2 | Formula 3 |
| Molds      | Control   | 5.32      | 5.07      | 5.25      |
|            | Beauveria bassiana | 4.93  | 4.05      | 4.79      |

Table 1. pH of liquid fermentation media with molds and yeasts after incubation
Our results showed the variation of pH shifting between inoculants in solid fermentation. The solid fermentation among the molds show variability. The acidity of media after fermentation with *Beauveria bassiana* was increased in both hydrolyzed and non-hydrolyzed media, compared to the control. On the other hand, fermentation with *Paelomyces lilacinus* showed increasing alkalinity in non-hydrolyzed media and no changing of pH was observed in hydrolyzed media. Fungal fermentation has been known to produce and accumulate organic acids that provide ecological as well as physiological advantageous for fungi [9]. As our result, fungal fermentation condition changes the acidity of their environmental condition, thus the decreasing of pH. The ability to convert carbon source into organic acids is quantitatively and qualitatively various among fungi. A study of fungal biodiversity of molds showed that *Aspergillus* sp. produces high concentrations of organic acids such as oxalic, citric and gluconic acids that can increasingly drop the pH of their environment [10].

| Table 2. pH of solid fermentation media with molds and yeasts after incubation |
|---|---|---|---|
| Inoculants | Species | pH |
| | | Hydrolysis | Non-hydrolysis |
| Mold | Control | 2.84 | 3.52 |
| | *Beauveria bassiana* | 2.63 | 2.50 |
| | *Paelomyces lilacinus* | 2.85 | 4.56 |
| Yeast | Control | 5.49 | 4.90 |
| | *Candida utilis* | 6.35 | 5.20 |
| | *Candida lessepsii* | 5.67 | 5.66 |

3.2. Determination of reducing sugars after fermentation time.
Sugars as carbon source impact the fermentation process including the production of organic acids such as citric acids. Reducing sugars such as glucose and fructose were common and they are available carbon source for sustain the metabolism of microbes. Monitoring carbon source, in this case the total reducing sugars, used after incubation time was conducted through DNS method. Table 3 showed the estimation of reducing sugar concentration in fermented broth of mold and yeast in three different formulas. All fungi consumed the available reducing sugars in all formulas. However, molds were obviously used more reducing sugars than yeasts. Between molds, *Paelomyces lilacinus* utilized the sugars more than *Beauveria bassiana*.

The estimation of used reducing sugars by so fungal fermentation was shown in Table 4. Different results from liquid fermentation, yeasts were apparently utilized more reducing sugars than molds. The concentration of acid for hydrolysis of sorghum bagasse did influence the available reducing sugars. The hydrolysis of bagasse for mold fermentation used higher concentration of sulphur acid resulting higher concentration of reducing sugars than yeast fermentation. Addition of acids helped to break down the bond of complex sugars into the product of more simple sugars.

| Table 3. Reducing sugars estimation after liquid fermentation with molds and yeasts after incubation |
|---|---|---|---|
| Inoculants | Species | Formula 1 | Formula 2 | Formula 3 |
| Mold | Control | 19.03 | 26.11 | 18.75 |
| | *Beauveria bassiana* | 11.67 | 11.67 | 11.34 |
| | *Paelomyces lilacinus* | 10.13 | 11.28 | 8.06 |
| Yeast | Control | 19.03 | 26.11 | 18.01 |
| | *Candida utilis* | 15.59 | 18.95 | 18.57 |
3.3. Citric acid formation

After observation of level of acidity and concentration of reducing sugars after incubation period, the citric acid concentration was measured through HPLC method (Figure 1). Table 5 shows the concentration of citric acid in mold and yeast fermentation broth. For all formulas, the citric acid formation through liquid fermentation was higher in yeast than in molds. Except in formula 3 with the presence of amino acid arginine as nitrogen source, *Beauveria bassiana* have higher ability to produce citric acid than *Paelomyces lilacinus*. Supplementation of sorghum juice with micronutrients and macronutrients did not significantly change the ability of yeasts, *Candida utilis* and *Candida lessepsii*, to produce citric acids.

The concentration of sugars affects the citric acid formation. As comparison, the available reducing sugars in solid fermentation was also lower than in liquid fermentation (Table 4). A study showed that the increasing of sugar concentration up to 150 g/L yielded higher citric acid formation by *Aspergillus niger* [13], but reduction in citric acid production was observed when the sugar concentration was more increased due to the over growth of the fungal biomass, which resulted in increased viscosity of the medium. Molds produced more citric acids in hydrolyzed media than non-hydrolyzed media. Acids in hydrolyzed media helped to breakdown the complex carbohydrate into simpler and more available saccharide so that increase the citric acid formation.

![Image of HPLC chromatograms](image_url)

**Figure 1.** Representative HPLC chromatograms of citric acid (CA) in C18 Cosmosil (15 cm x 4.6 mm) column as stationary phase and methanol : NaH2PO4 .H2O 50 mM pH 2.4 (11 : 89) as mobile phase with flow rate 1 mL/minutes. The analyte was detected by using UV-Vis detector at 215 nm.
Table 5. HPLC analysis of citric acid concentration after liquid fermentation with molds and yeasts after incubation

| Inoculants | Species             | Citric Acid Concentration (mg/L) | Formula 1 | Formula 2 | Formula 3 |
|------------|---------------------|----------------------------------|-----------|-----------|-----------|
| Molds      | Beauveria bassiana  | 27.68                            | 43.44     | 22.69     |           |
|            | Paelomyces lilacinus| 14.79                            | 33.27     | 34.94     |           |
| Yeasts     | Candida utilis      | 47.21                            | 46.93     | 48.31     |           |
|            | Candida lessepsii   | 47.21                            | 46.93     | 48.65     |           |

Table 6. HPLC analysis of citric acid concentration after solid fermentation with molds and yeasts after incubation

| Inoculants | Species             | Reducing Sugars Concentration (mg/L) | Hydrolysis | Non-hydrolysis |
|------------|---------------------|-------------------------------------|------------|----------------|
| Molds      | Beauveria bassiana  | 2.53                                | 0.33       |                |
|            | Paelomyces lilacinus| 1.27                                | 0.54       |                |
| Yeasts     | Candida utilis      | 1.48                                | ND*        |                |
|            | Candida lessepsii   | ND                                  | 2.54       |                |

*Not Detected

Citric acid yield in solid fermentation is lower than liquid fermentation (Table 6). One of the main reasons is the lower available sugar concentration in solid substrate than in liquid substrate. The submerged technique is widely used for citric acid formation and it is roughly calculated that about 80% of the production globally is obtained by submerged fermentation [14]. However, solid fermentation has been an alternative method for citric acid production by utilizing agroindustry residues such as sugarcane bagasse [15] and cassava bagasse [16]. Liquid fermentation or submerged fermentation for the production of citric acids profess several advantages compared to solid state fermentation. Submerged fermentation yields higher citric acid formation, relatively lower cost of process, lower maintenance, and less contamination than solid state fermentation [17].

4. Conclusion
The production of citric acid in solid fermentation is lower than liquid fermentation. Compared to molds such *Beauveria bassiana* and *Paelomyces lilacinus*, yeasts (*Candida* sp.) have higher ability to produce citric acid. Both in solid and liquid fermentation, the type of media as well as the concentration of sugars as carbon source did influence the citric acid formation.

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