Enhanced acetic acid production from manalagi apple (*Malus sylvestris* mill) by mixed cultures of *Saccharomyces cerevisiae* and *Acetobacter aceti* in submerged fermentation

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Abstract. The production of acetic acid from Manalagi apple was studied using a mixed culture of *S. cerevisiae* and *A. aceti* by submerged fermentation technique. Determination of the best conditions for producing acetic acid was performed by stratified optimization with variations that were made on the concentration of the initial sugar addition to the medium (0%, 10%, 20% w/v), the ratio of the number of inocula *S. cerevisiae* and *A. aceti* (7:3, 1:1, 3:7), and agitation rate (80 and 160 rpm). All experiments were done by using the initial pH medium of 4.5 and incubated at room temperature (28±2°C) for 14 days. The concentration of reducing sugar, alcohol, acetic acid, and the pH were measured every 48 hours. The efficiency of sugar conversion to acetic acid with the addition of initial sugar 0%, 10%, and 20% were 233%, 46.6%, and 6.4% respectively after ten days of incubation. Overall, the result showed that the highest acetic acid was produced from Manalagi apple juice when no sugar was added, using seven parts of *S. cerevisiae* to three parts of *A. aceti* and agitation rate of 160 rpm on the tenth day of fermentation. Under these conditions, glucose conversion efficiency to acetic acid increased to 362%.

1. Introduction

Acetic acid was produced by the obligate aerobic acetic acid bacteria (AAB) from alcohol substrate, whereas alcohol is produced by yeast through sugar fermentation anaerobically. Microorganisms commonly used in the fermentation of alcohol were *Saccharomyces cerevisiae* and *Zymonas mobilis* [1,2]. Karsch et al. [2] stated that for industrial fermentation *Z. mobilis* seemed to be inferior to *S. cerevisiae* due to the lower biomass production of the bacterium by the decrease in pH. Meanwhile, AAB commonly used were *Acetobacter* and *Gluconacetobacter*, two AAB genera that oxidize ethanol more easily than sugars [3].

Acetic acid production occurred in two separated bioprocesses [4]. Ghosh et al. [1] performed acetic acid production in two separated steps by adding sterile sugar and *A. aceti* starter culture after two days of ethanol fermentation by using *S. cerevisiae*, while Singh and Singh [4] adding inoculum *A. rancens* after 24 hrs. According to the characters of growth and metabolization of *S. cerevisiae* and *A. pasteurianus*, Wang et al. [5] conducted acetic acid process in three stages: the first stage for growth of *S. cerevisiae*, the second stage for co-culture of *S. cerevisiae* and *A. pasteurianus*, and the third stage for growth of *A. pasteurianus*. Generally, fermentation temperature and aeration rate were controlled.
differently at every stage [1][3]. Because it involved several different processes, the operational procedures of acetic acid fermentation became complicated and required a longer time.

In this work, acetic acid fermentation was done by using Manalagi apple, one of the apple cultivars found in Indonesia, as a substrate. In order to increase the production of acetic acid from Manalagi juice, this study performed stratified optimization with submerged fermentation using \( S. \text{cerevisiae} \) and \( A. \text{aceti} \) by determining the best conditions for producing acetic acid including initial sugar added to the medium, inocula ratio between \( S. \text{cerevisiae} \) and \( A. \text{aceti} \), and degree of agitation.

2. Methods

2.1. Microorganisms
The culture of \( S. \text{cerevisiae} \) ITBR58 and \( A. \text{aceti} \) ITBB24 obtained from culture collections of Microbiology and Fermentation Technology Laboratory of Chemical Engineering ITB. \( S. \text{cerevisiae} \) was cultured on a yeast extract glucose medium with a composition of 5 g of yeast extract, 10 g bacto peptone, 20 g glucose, 20 g bacto agar and 1000 mL distilled water. \( A. \text{aceti} \) was cultured on the same medium as \( S. \text{cerevisiae} \) but with the addition of 0.3 g of CaCO\(_3\) [6]. Based on the growth curve, the shortest generation time of \( S. \text{cerevisiae} \) was the 11th hour while \( A. \text{aceti} \) was the 9th hour (data not shown).

2.2. Fermentation medium
Manalagi Apple derived from Malang, East Java. The apple fermentation medium was prepared by juicing washed, filtered, and then pasteurized at 80°C for 30 minutes in three consecutive days.

2.3. Acetic acid production
Determination of the best conditions for producing acetic acid was performed by stratified optimization which included the initial sugar addition concentration, the ratio of the number of inoculum, and the speed of agitation. Variations that were made on the concentration of the initial sugar added to the medium were 0%, 10% and 20% in term of \%/v, the ratio of the number of inocula \( S. \text{cerevisiae} \) and \( A. \text{aceti} \) were 7:3, 1:1 and 3:7, and agitation rate of 80 and 160 rpm. In the first step, determination of the best initial sugar added to the medium was done by setting the ratio of 1:1 inoculum and 80 rpm of shaking speed. Next, the best number of inoculum ratio was determined by using the amount of initial sugar addition of the first stage result and 80 rpm of agitation. The last step, the best agitation rate was determined by using the best amount of initial sugar addition and the best ratio of inoculum of the second stage results. At each stage, the total number of inoculum of the mixed culture inoculated into the fermentation medium was 10% (\%/v) of the amount of fermentation medium. Overall optimization step and process for enhanced acetic acid production from Manalagi apple can be seen as below given (figure 1).

All fermentation processes were carried out in submerged batch fermentation in a 250 mL Erlenmeyer flask with a one-third work capacity and incubated at room temperature (28 ± 2°C) for 14 days. Reducing sugar content, alcohol, acetic acid and pH value were checked every 48 hours. Reducing sugar content determined by Somogyi-Nelson method [7][8]. Determination of alcohol and acetic acid content were done by distillation and titrable acidity methods respectively [9]. The conversion efficiency of sugar to acetic acid was calculated by the formula according to Kondo and Kondo [10]. Furthermore, the performance of the treatment with different agitation rates was evaluated by ANOVA with repeated measures using Minitab 17.0 statistical software.
Figure 1. The overall optimization steps for increasing the production of acetic acid from Manalagi apple using mixed cultures of *S. cerevisiae* and *A. aceti*.

3. Results and Discussion

3.1. Determination of the best initial sugar concentration added to the medium

Determination of the best initial sugar concentration added to the medium was intended to know the amount of sugar needed by *S. cerevisiae* and *A. aceti* as the substrate during acetic acid fermentation. Changes in reducing sugar content, alcohols, acetic acid and pH values during fermentation on the variations of initial sugar added to the medium can be seen in figure 2. The best initial sugar concentration added to the fermentation medium was determined by conversion efficiency of sugar to acetic acid in the treatment with various concentrations of initial sugar addition (table 1). According to table 1, the highest efficiency of sugar conversion to acetic acid was the addition of 0% initial sugar or no sugar added to the medium. Thus, these results will be used in the next stage of optimization.

| The percentage of the amount of initial sugar added to the medium (%) |
|-------------------------|---|---|---|
| 0%                     | 233% | 10% | 46.60% | 20% | 6.40%|

Table 1. The efficiency of reducing sugar conversion to acetic acid in fermentation condition with inocula ratio of *S. cerevisiae* and *A. aceti* 1:1, agitation rate 80 rpm, and room temperature for 10 days incubation.

Figure 2 showed the activity of *S. cerevisiae* and *A. aceti* and changes in reducing sugar content, alcohol, acetic acid and the value of pH medium during acetic acid fermentation process for all treatments. At the beginning of the fermentation process, a decrease in reducing sugar levels accompanied by an increase in alcohol levels. This indicates the presence of *S. cerevisiae* activity in hydrolyzing glucose to ethanol. Subsequently, there was a decrease in alcohol levels followed by an increase in acetic acid levels. This shows the activity of *A. aceti* in oxidizing ethanol to acetic acid.
Figure 2. Changes in reducing (a) sugar content, (b) alcohol, (c) acetic acid and (d) pH value. on the variation of initial sugar addition at fermentation condition with inocula ratio of *S. cerevisiae* and *A. aceti* 1: 1, agitation rate 80 rpm, and room temperature for 14 days incubation.

The results showed that the higher the initial sugar concentration added, the higher the reducing sugar content present in the medium and the higher the residual reducing sugar level at the end of the fermentation process (14th day) (figure 2a). In all treatments, especially in the addition of 10% and 20% starting sugar, the reducing sugar concentration decreased rapidly in two days of fermentation due to the high activity of *S. cerevisiae* in hydrolyzing sugar to alcohol. It was characterized by a high increase in alcohol on day 2 (figure 2b). The resulting alcohol contents at the initial sugar concentration of 10% and 20% had almost the same values accounted for 14.44% and 15.92% respectively. The alcohol content produced in both treatments was higher than the alcohol content produced at the treatment without the initial sugar addition of 6.91%. Thus, it can be inferred that the higher the initial sugar content was added the higher the alcohol content was produced. However, the alcohol content of the addition of 20% initial sugar treatment was very small when compared to the addition of 10% initial sugar treatment. This can be caused by the sugar content available in the medium with the addition of 20% being excess. Meanwhile, excess sugar in a medium will not increase microbial activity above its maximum threshold even the biomass of yeast produced can be limited to high sugar concentration [11].

From figure 2c, it can be seen that on the second day, in general, the higher the initial sugar concentration the higher the acetic acid content formed, except in the treatment with the addition of 20%
initial sugar. This was probably caused by high levels of sugar employed so that the environmental conditions become anaerobic or microaerobic where growth and activity of *A. aceti* which was aerobic obligate bacteria became inhibited, while *S. cerevisiae* grew well and produced high alcohol when compared with other treatment. On the treatment without the addition of the initial sugar, acetic acid produced with a high enough level of 4.44% in a relatively shorter time that was the sixth day compared with other treatments. However, during the fermentation process, the highest acetic acid level was produced by the treatment with the addition of 10% initial sugar which was 4.58% on the 12th day.

During the fermentation process, there was a tendency to change the pH of the medium (figure 2d). It indicated that in all treatments there was a change of sugar to acetic acid. Furthermore, the best initial sugar concentration in the fermentation process was determined based on the efficiency value of sugar change to acetic acid. Based on table 1, the efficiency of sugar conversion to the highest acetic acid was produced by the fermentation process with the treatment without the initial sugar addition. The value of treatment efficiency was more than 100%. This was due to the presence of hydrolysis of starch contained in substrates both chemically due to the decrease in pH and hydrolysis of oligosaccharides by *S. cerevisiae* for growth and metabolism activity [12][13][14]. Based on this, to conduct subsequent fermentation of acetic acid from apple juice was not required the addition of early sugar, because *S. cerevisiae* was able to decompose and utilize the sugar that already exists in apples. In other words, the nutrient amount contained in the apple was enough so that microbes can perform its maximum activities.

3.2. Determination of the best inoculum ratio of *S. cerevisiae* and *A. aceti*

Determination of the best inoculum ratio of *S. cerevisiae* and *A. aceti* was carried out with variations of the ratio of 3:7, 1:1, and 7:3 under conditions without the addition of initial sugar. Changes in acetic acid content and pH value during fermentation can be seen in figure 3. Figure 3 showed that the highest acetic acid concentration with the lowest pH value was achieved by mixed inocula of *S. cerevisiae* and *A. aceti* with a 7:3 ratio at day 8. These results were then used in the next stage.

The determination of the best inoculum ratio of *S. cerevisiae* and *A. aceti* aimed to evaluate the dominant factor of each microbe in a mixed culture in the fermentation of acetic acid. According to figure 3, it can be seen that all treatments produced high acetic acid levels. At the beginning of fermentation (day 0 to day 6), the highest acetic acid content was produced by treatment with the ratio of the inocula of *S. cerevisiae* and *A. aceti* 3:7. This can be due to the dominance of *A. aceti* to *S.
cerevisiae so that A. acetii can quickly utilize the glucose contained in the medium as its carbon source and convert the ethanol produced by S. cerevisiae to acetic acid. According to Maier [15], the initial of inoculum size will control the length of the lag phase. However, at a later stage, the resulting acetic acid content decreased. This can be due to the high amount of A. acetii that were requiring more nutrients. Limited nutrients available to A. acetii can cause cell growth and enzyme activity became not maximal, so the metabolic process of A. acetii did not work properly and the resulting acetic acid level became low. Moreover, acetic acid already produced will undergo overoxidation by A. acetii via TCA cycle [16].

Overall, the highest level of acetic acid was achieved by the treatment with a ratio of 7:3 inoculum on day 8. At the beginning of fermentation, the level of acetic acid concentration was lower than the treatment with the 3:7 inocula ratio. This can be caused by the dominance of S. cerevisiae. Under aerobic conditions, S. cerevisiae can still produce alcohol even in small amount. However, because of a large number of cells, the resulting alcohol can still meet the nutrient requirements for A. acetii. On the other hand, under these conditions, A. acetii can consume oxygen fully so that it can grow and adapt. At a later stage, the resulting acetic acid content became high. This can be due to the increasing activity of A. acetii and the availability of nutritional sources.

3.3. Determination of the best agitation rate
In the third stage, the best agitation rate determination was performed with variations of 80 and 160 rpm. The fermentation process was carried out under the condition without adding sugar to the ratio of inocula S. cerevisiae and A. acetii of 7:3. The percentage of acetic acid produced from both treatments can be seen in figure 4. Determination of the best agitation rate was intended to see the effect of agitation on growth and activity of S. cerevisiae and A. acetii in fermentation. Figure 4 showed that the faster the agitation the higher the resulting acetic acid content consistently. The highest acetic acid level reached an agitation rate of 160 rpm on day 10 in the amount of 6.47%. Agitation in a subsurface fermentation process aimed to increase the oxygen availability and solubility to the medium evenly. Thus, the aeration became better and the growth and activity of A. acetii cells became maximal. A. acetii can directly use dissolved oxygen to grow and produce acetic acid, and at the same time, the environment became anaerobic or microaerobic so S. cerevisiae can work to produce alcohol which was then used by A. acetii as a substrate to produce acetic acid. Therefore, a rise in agitation rate may lead to the production of acetic acid in a shorter time with a greater amount. With the optimization of the ratio of the inocula and the agitation rate, the efficiency of reducing sugar conversion to acetic acid increased to 362%. The result of variance analysis by repeating the measurements to the treatment with different shake speeds indicated that there was a significant difference.

3.4. Dynamics of changes in reducing sugar, alcohol, acetic acid contents and pH value during fermentation under optimum conditions
The dynamics of changes in the content of reducing sugar, alcohol, acetic acid, and pH value during fermentation of acetic acid from apple juice on the best conditions can be seen in figure 5. The figure showed that at the beginning of the fermentation process when the reducing sugar level was high, S. cerevisiae worked to produce alcohol so that the alcohol content became increased. After maximum alcohol level was reached, A. acetii seemed to start producing acetic acid so that acetic acid level began to increase. This goes on simultaneously. S. cerevisiae produced alcohol optimally while A. acetii performed growth activity and adaptation until the alcohol content produced by S. cerevisiae was sufficient for its nutrients. In the mixed culture, A. acetii which was aerobic obligate microbe used dissolved oxygen for growth and performed its activity in oxidizing alcohol to acetic acid, at the same time there was a condition where the medium was in anaerobic state due to lack of oxygen so that S. cerevisiae can perform its activity in converting sugar into alcohol. Another advantage of this mixed culture was the need for oxygen by A. acetii continuously causing S. cerevisiae to grow without the multiplication of cell mass. Thus, the sugar present on the substrate can be converted to alcohol by S. cerevisiae and subsequently to acetic acid by A. acetii maximally. At the end of the process, there was a decrease in the resulting acetic acid level. This may be due to an unfavorable pH of the medium for
microbes to be able to metabolize substrates and produce acetic acid as well as the acetic acid overoxidation due to the limited amount of nutrients.

4. Conclusion
The production of acetic acid from Manalagi apple with submerged fermentation technique has increased to the highest level in the condition of no sugar was added, using seven parts of _S. cerevisiae_ to three parts of _A. aceti_ and agitation rate of 160 rpm on the tenth day of fermentation.

5. References
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