Characteristics of Black Glutinous Rice Vinegar as Traditionally Fermented Product of Yeast Tapai and Acetobacter aceti

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Abstract. As the source of nutrition in food, the product of rice vinegar also has advantages as anti-diabetes, anti-oxidation, anti-cholesterol and anti-bacterial which contribute to health. For the current research, the fermentation of black glutinous rice for producing rice vinegar used microbes contained in yeast tape and acetic acid bacteria (BAA). To get high-quality vinegar products, two stages of the optimization process are carried out. The first process is the fermentation process for the production of alcohol using yeast from tapai and the second process is a fermentation process for the production of acetic acid using Acetobacter aceti. The products are analyzed for their physicochemical and functional properties. Vinegar produced in the processes contained 52.04 g/L acetic acid, 21.83% anthocyanin level, 1844.72 (GAE mg / L) total phenolic and 92.71% DPPH inhibition.

1. Introduction
Vinester is a liquid acid derived from the fermentation process of sugar or starchy ingredients into alcohol and then transform into acetic acid (vinegar). Vinegar is usually used in the food processing industry, chemical industry, and the pharmaceutical industry. Moreover, vinegar is also used as an alternative solution for health problems. Several studies which indicated the therapeutic properties of vinegar include antibacterial activity, reduction in blood pressure, antioxidant activity, reduction in the effects of diabetes, and prevention of cardiovascular disease. Other positive effects of consuming vinegar daily are reported to improve blood glucose responses that are beneficial for diabetic patients [1].

Vinegar is produced in two stages of fermentation. First is anaerobic fermentation uses yeast. The second process is aerobic acetic acid fermentation by Acetobacter aceti which oxidizes alcohol to acetic acid. Both fermentation is usually carried out separately [2]. According to [3], yeast is a starter used in the tapai fermentation process which consists of species of genera Aspergillus, Saccharomyces, Candida, Hansenulla, and bacteria Acetobacter. These genus lives together where Aspergillus simplifies starch to glucose and produces glucoamylase enzymes which decompose the starch by releasing glucose units, while Saccharomyces, Candida, and Hansenulla can decompose sugar into alcohol and various other organic substances. Meanwhile, Acetobacter decomposes alcohol into acid. According to [4], the fermentation process will produce a liquid which consists of water, acid, alcohol, and esters.

The chemical process of transforming sugar into alcohol, and alcohol to acidic is described in Figure 1.
2. Materials and Methods

2.1. Implementation of Research

Black glutinous rice vinegar mainly contains nutrients that are the same as the essential ingredients of starch, protein, minerals, and water. The strength of black glutinous rice compared to white rice lies in the skin of the seed. Beside rich of natural fiber and oil, black glutinous rice skin also contains anthocyanins which can improve the functional properties of vinegar product.

2.1.1. Tapai fermentation

Black glutinous rice tapai is made using tape yeast. Treatment is A. type of rice (A1) cracked skin glutinous rice, (A2) glutinous rice polished, (B1) Treatment B = yeast concentration (B1 = 2%; B2 = 4% and B3 = 6%). The fermentation time is up to 72 hours, in anaerobic conditions. After that, the observation is done toward water content, sugar content, alcohol content, total acid, pH, total anthocyanin, total phenol, and antioxidant activity.
2.1.2. Vinegar Fermentation

The fermented tapai of black glutinous rice which meets the requirements is fermented with Acetobacter aceti starter. Treatment A is adding water (A1 = without addition; A2 = 10% of the weight of tape; A3 = 20% of the weight of tape; A4 = 30% of the weight of tape). Treatment B is the concentration of BAA (B1 = 0%; B2 = 1%; B3 = 2% and B4 = 3%). The duration of fermentation is 20 days in aerobic conditions. After fermentation, the observations proceed to the analysis of water content, sugar content, alcohol content, total acid, and pH. For the best results, the analysis carries forward to the profile of organic acids, total anthocyanin, total phenol, antioxidant activity, etc.

2.2. Observations

The observations made are: Water Content Analysis with Oven Method [8], Total acid analysis titrated with titration methods [9], Analysis of Alcohol Levels [10]; Analysis of Total Sugar Levels with the Luff Schrool Method; pH Determination Analysis with pH-meter tool; Analysis of Anthocyanin Levels with Differential pH Methods [11]; Phenol Level Analysis of the methods of [12]; Analysis of Antioxidant Activity with DPPH [13].

3. Results and Discussions

3.1. Characterization of Tapai

The fermented black glutinous rice with yeast tapai on the characteristics of black glutinous rice such as water content, sugar content, alcohol content, titrated acid total and acidity can be seen in Table 1.

| Parameter                        | The combination of black sticky rice and yeast tapai |
|----------------------------------|------------------------------------------------------|
|                                  | A1B1 | A1B2 | A1B3 | A2B1 | A2B2 | A2B3 |
| Water content (%)                | 57.53±0.56 | 58.08±0.46 | 59.73±0.32 | 60.81±0.26 | 61.18±0.20 | 62.28±0.24 |
| Reducing sugar levels (mg/100 ml) | 14.99±0.94 | 11.96±0.91 | 9.54±0.13 | 15.62±0.33 | 12.80±1.55 | 9.95±0.68 |
| Alcohol content                  | 1.80±0.16 | 2.05±0.13 | 2.34±0.33 | 2.86±0.19 | 3.17±0.20 | 3.45±0.10 |
| Total titrated acid (mg / 100 ml) | 2.56±0.14 | 2.80±0.14 | 2.80±0.14 | 3.44±0.37 | 3.52±0.37 | 3.76±0.28 |
| Degree of acidity (pH)           | 4.53 | 4.37 | 4.23 | 4.00 | 3.90 | 3.90 |
| Anthocyanin level (mg / 100 ml)  | 5.86±0.91 | 4.8±0.09 | 4.3±0.09 | 3.84±0.18 | 2.63±0.09 | 2.38±0.09 |
| Total phenolic (GAE mg/ L)        | 1366.39±6.68 | 1268.61±6.25 | 1227.22±3.76 | 1167.22±4.19 | 1149.44±2.93 | 1035.00±5.83 |
| Percent DPPH inhibition           | 88.27±0.21 | 88.10±0.10 | 87.56±0.12 | 86.00±0.26 | 86.15±0.27 | 85.50±0.41 |
| Total Plate Number (colony / ml)  | 6 x 104 | 8 x 104 | 4 x 104 | 6 x 104 | 3 x 104 | 6 x 104 |
| Staphylococcus aureus             | Negative | Negative | Negative | Negative | Negative | Negative |
| Mold and yeast (colony / ml)      | 5 x 103 | 3 x 103 | 3 x 103 | 6 x 103 | 3 x 103 | 6 x 103 |

Description: (A1) cracked skin glutinous rice, (A2) glutinous rice polished, (B1) yeast 2%, (B2) yeast 4%, (B3) yeast 6%.

Water content and sugar content of black glutinous rice are increased during the fermentation process, so alcohol and acid are also formed. It is because the microorganisms in yeast decompose the starch into sugar and then become alcohol and then transform the alcohol to acetaldehyde and water so
that the water content in the tapai is increased. According to [4], the fermentation process will produce a liquid consisting of water, acid, alcohol, and esters. Hence, the liquid formed from the fermentation process will affect the increase of water content in the material.

Black glutinous rice as a raw material contributes as a source of natural antioxidants to the products because it contains anthocyanin and several other phenol compounds. The results of Table 1 show that DPPH inhibition is quite high 88.27 per ml of tapai extract, while the anthocyanin content is 5.86 mg / 100 ml. The results of the research by [6], showed that the highest DPPH inhibition was 92.30% and the anthocyanin content was 4.24 mg / 100 ml, the raw material used was black cracked glutinous rice, namely rice which still had a husk. The difference in antioxidant activity and anthocyanin content of the two studies can only be caused by different processing conditions and execution times, besides that antioxidant activity can also be influenced by other components contained in the raw material.

The microbial observations based on the total plate number showed that the maximum number was 8 x 10^4 colonies/ml, molds and leaves were 6 x 10^3, while the Staphylococcus aureus bacteria were negative. It shows that the fermentation process is still running. During the process, there are still changes to the organic substrate found in the raw material, i.e. black glutinous rice which has been previously steamed. Likewise, microbes from tape yeast still regenerate.

The black glutinous rice tapai is one of the probiotic foods, i.e. foods containing non-pathogenic living microbes and actively useful to improve health by maintaining the balance of microbes in the intestine. The black glutinous rice contained microbes which are dominated by mold, yeast and acetic acid bacteria and lactic acid [14]. In addition, black glutinous tapai also contains anthocyanin as a source of red blue and purple colour which is a component of black glutinous rice flavonoids that have phenolic compounds and are bioactive compounds that have antioxidant activity, are cholesterol-lowering and anti-tumor [15] and can be used as natural dyes in food and beverages [7].

3.2. Characterization of Rice Vinegar
The second stage of fermentation was carried out on the fermented tapai from the treatment of polish rice with yeast reaching 6%. It is used because of the highest water and alcohol content. In vinegar fermentation by acetic acid bacteria is a substrate containing alcohol, besides that high water content will also provide a good condition for the expected bacterial growth. The combination of treatments in this study was the addition of water to the substrate (tapai) used and the use of starter acetic acid bacteria; the amount added each of which was a percentage of the substrate. The results of the analysis of the characteristics of the vinegar produced are presented in Table 2.

The results of the analysis shown in Table 2 are there is a correlation between the addition of water and the AAB starter to the water content, total reducing sugars, alcohol content, acetic acid level and acidity (pH) of the product produced. The level of water increases because of the alcohol change into an acetaldehyde. Besides that, it is also due to the addition of water to the substrate for fermentation. The tapai fermentation process still occurs because all the microbes present in the yeast work in symbiosis and are supported by the nutrient, water, pH and oxygen content of the very supportive substrate. This process can be seen in the data in Table 2, where the reduced sugar content decreases, the total alcohol content increases as a result of the sugar reshuffle by S. cerevisiae, as well as the acid level increases as a result of the alcohol turnover into acetic acid by A. aceti.

According to [16], many factors influence fermentation including substrate, temperature, pH, oxygen, and microbes used. The substrate is a fermentation raw material that contains nutrients needed by microbes to grow and produce fermented products. A nutrient which is most needed by microbes both to grow and to produce fermented products is carbohydrate. The substrate used in this study was black glutinous rice. According to the Ministry of Health's Directorate of Nutrition, black glutinous rice (per 100 gr) has a carbohydrate content of 78 gr. Carbohydrates contained in black glutinous rice are used as a producer of energy for microbial growth in yeast. According to [17], the longer the fermentation time, the more glucose is transformed into alcohol. Therefore, the alcohol content produced is higher then the alcohol will be transformed into acetaldehyde and acetic acid.

According to the Codex standard [18], the limit of acetic acid in vinegar is at least 50g/ L, while the residual alcohol is a maximum of 10%, dissolved solids are at least 2g / L. Similarly, according to
the National Standardization Agency (BSN) [19], in SNI 01-4371-1996 the quality requirements for fermented vinegar are the minimum limit of the amount of acetic acid is 4%, the minimum sugar content is 15% and the remaining alcohol is a maximum of 10%.

The results of the study in Table 2. have met the requirements for acetic acid and residual alcohol levels, but for sugar content is still low. It is important to consider the environmental conditions during the fermentation process, especially the oxygen supply. Vinegar fermentation with A. aceti must be in aerobic with sufficient oxygen. When oxygen is less, then the alcohol fermentation by S. cerevisiae continues to reform and produces alcohol. Whereas alcohol which is formed imperfectly is transformed into acetic acid by AAB. To catalyze the reactions that give them energy, acetic acid bacteria need an adequate and sustainable supply of oxygen in all parts of the tank. Oxygen supply will affect performance [1; 20]. In the fermentation process submerged in liquid, this, acetic acid bacteria will attract energy from the oxidation of ethanol to acetic acid

| Treatment | Water Content (%) | reducing sugars (%) | Alcohol level (%) | Level of acetic acid (g/L) | Degree of acidity (pH) |
|-----------|-------------------|---------------------|------------------|--------------------------|------------------------|
| A1B1      | 55.76±0.19        | 6.85±0.19           | 3.75±0.02        | 28.02±1.73               | 4.07                   |
| A1B2      | 56.39±0.32        | 5.90±0.54           | 4.41±0.08        | 31.03±1.73               | 3.80                   |
| A1B3      | 57.43±0.05        | 5.30±0.44           | 5.20±0.04        | 32.03±1.73               | 3.77                   |
| A1B4      | 58.20±0.13        | 3.10±0.07           | 5.74±0.04        | 35.03±1.73               | 3.73                   |
| A2B1      | 65.47±0.40        | 6.35±0.04           | 4.76±0.08        | 32.03±1.73               | 3.63                   |
| A2B2      | 66.51±0.49        | 5.50±0.04           | 5.55±0.04        | 35.03±1.73               | 3.77                   |
| A2B3      | 67.10±0.04        | 3.80±0.21           | 6.16±0.17        | 38.03±1.73               | 3.77                   |
| A2B4      | 68.33±0.34        | 2.10±0.08           | 6.60±0.17        | 41.03±1.73               | 3.67                   |
| A3B1      | 75.47±0.45        | 5.75±0.07           | 5.20±0.19        | 37.03±1.73               | 3.60                   |
| A3B2      | 76.37±0.39        | 4.05±0.01           | 5.94±0.13        | 40.03±1.73               | 3.67                   |
| A3B3      | 77.43±0.11        | 2.30±0.13           | 6.67±0.29        | 43.04±1.73               | 3.67                   |
| A3B4      | 79.41±0.33        | 1.75±0.14           | 7.13±0.50        | 45.04±1.73               | 3.63                   |
| A4B1      | 85.33±0.32        | 4.40±0.09           | 5.34±0.23        | 38.03±1.73               | 3.63                   |
| A4B2      | 86.50±0.23        | 2.80±0.23           | 6.11±0.08        | 43.04±1.73               | 3.57                   |
| A4B3      | 85.33±0.32        | 4.40±0.09           | 5.34±0.23        | 38.03±1.73               | 3.63                   |
| A4B4      | 85.33±0.23        | 2.80±0.23           | 6.11±0.08        | 43.04±1.73               | 3.57                   |

Description: A: addition of water: A1 = 0; A2 = 10%; A3 = 20%; A4 = 30%.
B: Acetobacter aceti starter: B1 = 0; B2 = 2%; B3 = 4%; B4 = 6%

3.3. Recommended rice vinegar

The results of the combination treatment of vinegar production in this study recommend adding 30% water to the tape and using AAB 6% as a starter in fermentation. In addition to the parameters in Table 2, it was also observed the yield formed, total anthocyanin, total phenol and antioxidant activity and components contained in the vinegar with GC-MS tools. The vinegar characteristics that are produced can be seen in Figure 2 and Table 3.
There is no acetic acid standard for the identity of the quality of rice vinegar. In Brazil, the regulations are only for wine vinegar, fruit, alcohol, and alcohol cereal. Based on the quality standards needed for the type of vinegar mentioned, rice vinegar, the quality standard is the content of acetic acid. Acidity by determining whether the product is vinegar or dilute acetic acid. Several tests have been done with high costs [22]. According to [23] a simple method, but not yet fully reliable, is pH measurement. Non-volatile buffer substances have higher pH values than those observed in acetic acid solutions with equivalent concentrations.

Based on Table 3 and Figure 2 it can be seen that the results of this study are black glutinous rice vinegar which has functional properties in the presence of anthocyanin and phenolic compounds which are equivalent to gallic acid with high content and high antioxidant activity in inhibiting DPPH radical compounds.

According to [1], vinegar is an acidic spice with various functional properties, including antibacterial, anti-infective, antioxidant, anticancer activities, blood glucose control, regulation of lipid metabolism, and weight loss. The antibacterial and anti-infectious effects of vinegar are mainly due to the presence of organic acids, although polyphenols and melanoidins in some vinegar also contribute to this property. Antioxidant ability of vinegar mainly comes from polyphenols and melanoidins, which are influenced by raw materials and fermentation conditions, respectively. The effect of some vinegar on blood glucose control, lipid metabolism regulation, and weight loss is due to the presence of acetic acid, which is mainly produced by acetic acid bacteria during fermentation.

4. Conclusions
Vinegar requires the demand for industrial fermentation systems that are capable of producing large amounts of vinegar. This system must maintain reliable control and optimum conditions for alcoholic fermentation from tapai and acetic acid bacterial fermentation. The specialty of vinegar from black sticky rice in addition to high acetic acid is the anthocyanin content and phenol compounds. Vinegar
produced has antioxidant activity and other functional properties. It is recommended for further research to determine the optimization of the acetic acid fermentation process to reduce residual alcohol. And also observe other functional properties.

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