The variation of ethanol concentration and kombucha characterization on several incubation periods

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Abstract. Kombucha is a type of fermented tea that recently popular in Indonesia. It gives so many health benefits for human body, as prevention and therapeutic cure for the disease. Some of the benefits are for improving digestion and reducing the risk of cancer and cardiovascular disease. It also has significant potential as an antioxidant agent. The heterofermentative fermentation process in kombucha causes this product to produce organic acid and ethanol as an alcohol compound. However, ethanol concentration in fermented food has become a major concern in Indonesian Halal products studies. One cause of ethanol concentration differences is incubation times. In this study, we measured alcohol concentration at several incubation times: 5, 6, 7 and 8 days incubation. We also assessed characteristics of kombucha by testing its antioxidant activity, lactic acid content, pH, and enumeration of lactic acid bacteria. The result shows that the incubation time, from 5 to 8 days, did not produce alcohol more than 0.5% as restricted by MUI Fatwa 2018. It has been shown that the various time of incubations did not give a significant difference in the quality of the antioxidant activity and lactic acid levels, and pH (p>0.05).

1. Introduction
Kombucha is a fermented tea that provides a lot of benefits for human health: reducing the risk of cancer, cardiovascular disease, improving digestion and oral health, stimulating the immune system, reducing inflammation, detoxification, and source of the antioxidant agent [1], [2], [3]. Most of the benefits acquired by many kinds of probiotics and its secondary metabolite products: gluconic acid, ethanol, acetic acid, phenolate acid, enzyme, phenolic substance, and vitamin B [4].

Tea is fermented by hetero-fermentative bacteria and yeasts that produce some organic acids and ethanol as an alcohol compound [5]. The presence of ethanol in kombucha has become a controversy in Indonesian Halal products studies. The Council of Indonesian Ulama (MUI) Fatwa no.10 of 2018 concerning about Food Products and Beverages Containing Alcohol/Ethanol restricts the percentage of ethanol in a beverage that must be less than 0.5% and not harmful to human health [6]. The duration of incubation times determines the level of ethanol in the fermentation product. It can be rising along with the times of incubation. Therefore, in this study, we measured alcohol concentration of kombucha at several times of incubation. The lowest of alcohol content that remained a good characteristic of kombucha will become an alternative solution of halal product. It also contributed to determining the critical halal point of kombucha.

2. Experimental
2.1. Fermentation
Green tea was obtained from Kina and Tea of Research Institution, Ciwidey, Indonesia. Symbiotic Culture of Bacteria and Yeast (SCOBY) from Wikikombucha Bandung, Indonesia was used as a starter culture of kombucha. Green tea was fermented using SCOBY following a protocol from Wikikombucha [7] with modification. Boiled water was mixed with 15 g/L of green tea leave. The solution was filtered out, and 11% (w/v) sugar was added into it. The fermentation process was performed in 500 mL glass jar. The mixture of tea water and sugar was cold down in room temperature. SCOBY was added to the solution. The glass jar was covered by cotton, then incubated in room temperature without sunlight. The incubation times were 5, 6, 7 and 8 days incubation.

2.2. Determination of Alcohol Concentration
A 50 mL kombucha samples were distilled to obtain pure alcohol. The concentration of ethanol was measured using pycnometer with serial ethanol standard curve: 0,1%, 0,2%, 0,4%, 0,8%, and 1,6%. The ethanol concentration of every sample was determined using a regression equation based on the alcohol standard curve.

2.3. Determination of Lactic Acid Bacterial (LAB)
LAB was determined using the Total Plate Count (TPC) technique on Man Rogosa and Sharpe (MRS) culture media according to Fardiaz method [8].

2.4. Determination of pH and Organic Acid Compound
The acidity of every sample was measured by using pH meter. Measuring of lactic acid as the organic acid compound was conducted by acid-base titration using 0,1 N oxalate acid and 0,1 N NaOH as standard solutions.

2.5. Determination of Antioxidant Activity
Antioxidant activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) method with butylhydroxytoluene as standard. IC50 for each sample was determined using the regression equation based on the standard curve.

3. Result and Discussion
In kombucha fermentation process, tea is usually fermented for 7 until 10 days. Kombucha will have a new healthy layer of SCOBY on the surface of the solution. The taste is lightly sweet and mildly acidic [9]. In this research, a new healthy SCOBY was formed starting from the fifth day of incubation until the eighth day of incubation. The new SCOBY was formed as a thin floating layer on the surface of the solution. The colour was ivory and about 0,1 cm of the thickness (Figure 1). It was getting thicker along with the times of incubation. The solution had a brownish colour. The tastes were sour, lightly sweet, and a little bit bitter.
The bar chart in Figure 2 shows the rising of ethanol from 5th day to 7th-day incubation. It was caused by the activity of microbes/SCOBY, such as Saccharomyces cerevisiae that converted sugar to ethanol and CO$_2$ during alcohol fermentation [10]. However, the percentage of alcohol in 5th day to 8th-day incubation did not produce alcohol more than 0.5% ($p<0.05$) as restricted by MUI Fatwa 2018 [6]. Therefore, 5-8 days fermented tea can be considered as a halal beverage. Besides, the concentration of ethanol that more than 0.5% can also be of concern to infants and toddlers [11]. Public Health Agency of Canada (PHAC) also said even though there is no concern about the alcohol content in kombucha, but it should not exceed 0.5% [12].

Sugar concentration also contributes to the result of ethanol production. The added sugar in kombucha production is usually about 5-15% (w/v) [13]. In this research, sugar was added into the tea water by 11%. The level of ethanol has a positive correlation with the increase in sugar concentration until at a specific point. It is because sugar is a substrate for alcohol fermentation [14].
Table 1. Kombucha characterization on several incubation periods

| Incubation time (day) | Lactic acid concentration (%)<sup>a</sup> | pH<sup>a</sup> | IC<sub>50</sub><sup>a</sup> | LAB 10<sup>4</sup> (CFU/mL) |
|-----------------------|----------------------------------------|---------------|-----------------|----------------------------|
| 5                     | 0.33 ± 0.04                            | 3.70 ± 0.01   | 0.17 ± 0.03     | 5                          |
| 6                     | 0.40 ± 0.06                            | 3.63 ± 0.01   | 0.17 ± 0.06     | 19.5                       |
| 7                     | 0.33 ± 0.08                            | 3.37 ± 0.01   | 0.19 ± 0.01     | 49                         |
| 8                     | 0.39 ± 0.05                            | 3.27 ± 0.01   | 0.17 ± 0.04     | 1.2                        |

<sup>a</sup>p>0.05

The concentrations of lactic acid in several incubation periods were 0.33 – 0.4% without any significant difference (p>0.05), so do the degrees of acidity (pH) of kombucha (table 1). The production of lactic acid in kombucha contributed to the pH reduction that could be profitable for the growth of the LAB group, so that can avoid the pathogen microbe growth [15]. It was said if the pH does not reach pH ≤ 4.2 for 7 days incubation, kombucha is most likely contaminated [11]. However, it was reported at Iowa in 1995, kombucha could be harmful for consumption if fermented within 14 days incubation. The high concentration of lactic acid will cause severe metabolic acidosis [9].

The activity of antioxidant was determined using DPPH method. The activity of antioxidant was presented in IC<sub>50</sub>. A low number of IC<sub>50</sub> indicated a high activity of compound for binding to a free radical [16]. Polyphenol, ascorbic acid, and DSL are known as compounds that have the activity of antioxidant in kombucha. These activities were better than tea as a raw material [2]. Table 1 shows that there was no significant difference in IC<sub>50</sub> between all incubation periods (p>0.05). It was indicated that the 5 days incubation as the shortest incubation time also had a good potential of the antioxidant agent. Besides, it had the lowest concentration of ethanol, by around 0.02% (Figure 2).

The result of LAB determination shows that the amount of LAB colony increased from day-5 to day-7 of incubation, then it decreased on day-8 incubation (table 1). The highest of LAB colony was on 7 days of incubation, by 49x10<sup>4</sup> CFU. Lactic acid bacterial is probiotic bacteria that can give much beneficial for health, mainly to keep digestion and immune system. Probiotics get into the digestive tract can minimalize the pathogen microbes that live in the digestive canal [17]. Furthermore, LAB can produce various secondary metabolite products that give the benefits for human body: detoxification; reducing cancer risk, cardiovascular disease and inflammation [1][2][3].

4. Conclusion

The ethanol concentration on 5, 6, 7, 8 days incubation of kombucha were 0.02%, 0.16%, 0.32%, and 0.26%, respectively, with no significant difference in the quality of the antioxidant activity, lactic acid levels, and pH.

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