VOLUME 10 NOMOR 2 DESEMBER 2023

ISSN 2548 – 611X

JURNAL
BIOTEKNOLOGI & BIOSAINS INDONESIA

Homepage Jurnal: http://ejournal.brin.go.id/JBBI/index

THE EFFECT OF INCUBATION PERIOD ON ETHANOL CONTENT OF JASMINE AND GREEN TEA KOMBUCHA

Waktu Inkubasi terhadap Kadar Etanol pada Kombucha Teh Melati dan Teh Hijau

Nisa Ihsani, Nelis Hernahadini*, Zahra Salsabila Nurissalma, Annisa Nurul Huda, Tiwi Sartika, Arifa Nadya Nur Afifah, and Ninda Rahma Nur Syamsiyah

Department of Biotechnology, Universitas Muhammadiyah Bandung,
West Java 40614, Indonesia
*Email: nelis.hernahadini@umbandung.ac.id

ABSTRACT

Kombucha is a fermented tea that provides many benefits for health. The alcohol (ethanol) content of kombucha has withdrawn a lot of attention because of human health risks and halal concerns. Alcohol and Tax and Trade Bureau (TTB), BC Center for Disease, and MUI fatwa have defined that kombucha is safe to be consumed as long as the alcohol content is less than 0.5%. This study was conducted to compare the alcohol (ethanol) concentration of jasmine and green tea kombucha during the fermentative stage (5 day at 25°C) and post-fermentative stage (7 and 21 day, both at 4°C). The result showed that ethanol concentrations for jasmine and green tea kombucha rose from the fifth d of incubation to the seventh d of the post-fermentative stage, followed by a decline at 21th d of the post-fermentative stage (p>0.05). The level of organic acids, Acetic Acid Bacteria (AAB), and pH confirmed that there were no significant differences at all (p>0.05). In conclusion, jasmine and green tea kombucha at fermentative stage (5 d at 25°C) and post-fermentative stage (7 and 21 day, both at 4°C) did not produce ethanol more than 0.5%, without any significant differences in organic acids and AAB levels, respectively.

Keywords: *ethanol; fermentation; incubation; kombucha; tea*

ABSTRAK

Kombucha adalah teh fermentasi yang memberikan banyak manfaat bagi kesehatan. Kandungan alkohol (etanol) kombucha menarik banyak perhatian karena risiko kesehatan manusia dan masalah halalnya. Alkohol dan *Tax and Trade Bureau* (TTB), *British Columbia* (BC) *Center for Disease*, dan fatwa MUI telah menetapkan bahwa kombucha aman dikonsumsi selama kandungan alkoholnya kurang dari 0,5%. Penelitian ini dilakukan untuk membandingkan konsentrasi alkohol (etanol) melati dan teh hijau kombucha selama tahap fermentasi (5 hari pada suhu 25°C) dan tahap pasca fermentasi (7 dan 21 hari pada suhu 4°C). Hasil penelitian menunjukkan bahwa konsentrasi etanol untuk melati dan kombucha teh hijau naik dari hari kelima inkubasi ke hari ketujuh dari tahap pasca-fermentasi, diikuti oleh penurunan pada hari ke-21 dari tahap pasca-fermentasi (p>0,05). Tingkat asam organik, Bakteri Asam Asetat (AAB), dan pH menegaskan bahwa tidak ada perbedaan yang signifikan sama sekali (p>0,05). Kesimpulannya, melati dan teh hijau kombucha pada tahap fermentasi (5 hari pada 25°C) dan tahap pasca-fermentasi (7 dan 21 hari, keduanya pada 4°C) tidak menghasilkan etanol lebih dari 0,5%, tanpa perbedaan signifikan masing-masing dalam asam organik dan kadar AAB.

Kata Kunci: etanol; fermentasi; inkubasi; kombucha; teh

Received: *07 October 2023* ***Accepted***: *30 December 2023* ***Published***: *30 December 2023*

INTRODUCTION

Kombucha is a functional beverage made from tea and sugar through a fermentation process by Symbiotic Culture of Bacteria and Yeast (SCOBY). There are numerous beneficial substances in kombucha, such as organic acids, minerals, vitamins, flavonoids (Jakubczyk et al. 2020), antibacterial, polyphenols, and antioxidants. Kombucha provides many benefits for human health. It protects human body from lipid peroxidation due to the antioxidant substances (Lobo et al. 2017). Kombucha also has an anti-diabetic properties, stimulates detoxification of the liver, and reduces the level of cholersterol (Jakubczyk et al. 2020),

The final product of kombucha contains ethanol, which has become concern for halal issues (Ihsani et al. 2021). Halal means permissible and lawful life practices in Islam. Consuming halal food and beverage is an obligation that every muslim must follow as a part of the Islamic faith (HMC, 2024). It is forbidden in Islam to consume non-halal food or beverage as it is categorized as haram, such as khamr. Khamr is any intoxicating substance that causes the person losing their ability to control their mind and actions. The prohibition of drinking khamr is stated in Holy Quraan Surah Al-Baqarah verse 29 and Al-Maaidah verse 90. Ethanol as a by-product and derivative in the khamr industry is not permissible to drink. On the other hand, any food such as fruit containing natural alcohol and beverage that is processed without the intention to produce khamr under a certain percentage is permissible to drink (Arshad and Nur, 2018).

The level of ethanol in kombucha has to be considered due to the halal and health concerns. %bythey are Besides, the Tax and Trade Bureau (TTB) (2019) and (BC Centre for Disease Control 2020) stated that kombucha is safe to be consumed if only the alcohol (ethanol) content is less than 0.5 %. Drinking excessive alcohol can lead to the development of serious problem health and trigger many diseases: heart disease, cancer, dementia, mental health problems, digestive problems, and weakening of the immune system (CDC, 2022).

Neffe-Skocińska et al. (2017) reported that the alcohol (ethanol) content of kombucha could reach the maximum value of 1,1% during the ten days of incubation at 25^{o}C. Moreover, it is also said that some yeast and bacteria in kombucha could live at a low temperature as a storage temperature (Laureys et al. 2020). Thus, it is possible that alcoholic fermentation can continue at storage temperature. Therefore, the alcohol (ethanol) content of kombucha must be determined at the storage temperature on the early and onset post-fermentative stages. Therefore, this research was conducted to compare the alcohol (ethanol) concentration of jasmine and green tea kombucha during the fermentative stage (5 days at 25^{o}C) and post-fermentative stage (7 and 21 day, both at 4^{o}C).

MATERIAL AND METHODS

Place and Time of Research

The research took place at Biotechnology Laboratorium in University of Muhammadiyah Bandung. The study was conducted in March-September 2021.

Materials

Jasmine tea was obtained from PT Gunung Slamat, Indonesia, while green tea was obtained from Cinchona and Tea Research Institute, Indonesia. Boiled water, Sugar (PT Sugar Group Companies, Indonesia), GYC medium; yeast extract, glucose, calsium carbonate and agar, alcohol, Natrium Hidroksida, Oxalate acid.

Methode

Fermentation

Jasmine and green tea were fermented using Symbiotic Culture of Bacteria and Yeast/SCOBY *(Acetobacter xylinum, A. xylinoides, dan Bacterium gluconicum, Saccharomyces cerevisiae, S. ludwigii, Zygosaccharomyces bailii, Z. rouxii, Schizosaccharomyces pombe, Torulaspora delbrueckii, Brettanomyces bruxellensis, B. lambicus, B. custersii, Candida sp., and Pichia membranaefaciens)* following Adi (2018) and Ihsani et al. (2021) protocols. Each tea was added to boiled water as much as 15 g/L. The solution was mixed with 11% (w/v) of sugar and cooled down in a glass jar. SCOBY was added to the tea and sugar mixture then incubated (manual) at 25^{o}C for

5 days without sunlight. After five-day incubation, the samples were collected and analyzed to determine ethanol content, Asetic Acid Bacteria (AAB), pH, and the level of organic compounds. The remaining samples were separated with SCOBY and then stored at 4^{o}C. These samples were further collected and analyzed at the 7th and 21st day.

Determination of Alcohol (Ethanol) Level

Serial alcohol (ethanol) standard curve was made using pycnometer with concentrations: 0,1%, 0,2%, 0,4%, 0,8%, dan 1,6%. Each sample was distilled to obtain pure alcohol (ethanol), then the alcohol (ethanol) level was determined using a regression equation based on the ethanol standard curve.

Determination of Acetic Acid Bacteri (AAB)

AAB was selected by GYC medium containing 1% yeast extract, 10% glucose, 2% CaCO3, and 1,5% agar according to the method proposed by Gullo et al. (2006). Bacteria were cultivated in incubator for 2-5 d at 30^{o}C and counted using the Total Plate Count (TPC) technique.

Determination of Organic Acid Compound and pH

The organic acid compound was determined by acid-base titration using a burette with standard solutions: 0,1 N NaOH and 0,1 N oxalate acid. A pH meter was used for measuring the acidity of every sample.

RESULT AND DISCUSSION

A new healthy layer of SCOBY was formed as a biofilm on the surface of kombucha after 5 d incubation (Figure 1A). Both of jasmine and green tea kombucha produced an ivory-coloured SCOBY layer with a thickness of around 0,1 cm. This cellulose layer was produced by *Acetobacter xylinum* (Ross et al. 1991). The formation of the cellulose started from the synthesis of Uridine Diphospho-Glucose (UDPGlc) as a cellulose precursor. Then, it polymerized into β-1,4-glucan chains (Villarreal-Soto et al. 2018). These chains would compose a new layer of SCOBY (Saxena and Brown 2005) that allowed the association of bacteria and yeast to obtain atmospheric oxygen (Ross et al. 1991). Therefore, bacteria and yeast could grow and produce more cellulose from the various carbon sources (Villarreal-Soto et al. 2018).

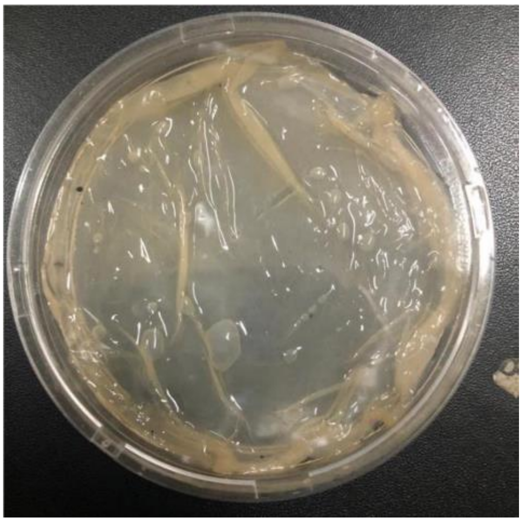

Figure 1. A layer of SCOBY after 5 days incubation of kombucha fermentation at 25^{o}C.

All of the kombucha alcohol (ethanol) contents at different stages were below 0,5% (p>0,05%). It was due to the short time of incubation. However, the alcohol (ethanol) levels of jasmine and green tea kombucha increased from the fermentative stage (day 5 at 25ºC) to the post-fermentative stage (day 7 at 4ºC) (Figure 2). It indicated that the remaining microbes grew for one week at the storage temperature in order to perform alcoholic fermentation. Some alcohol fermenting microbes in SCOBY can tolerate low temperatures. S. cerevisiae has an optimal temperature range of 25-30 ºC, but can adapt to environmental changes and maintain its viability at 4ºC for a long time (Crawford and Pavitt 2019; Liszkowska and Berlowska 2021). The ethanol concentration that drops at the beginning of the post-fermentation stage is caused by the storage process at 4ºC which is not the optimal temperature for SCOBY to grow up. (Figure 2). Tan et al. (2020) also reported that all microorganisms in kombuca decreased from day 14 to day 21 of incubation at a storage temperature of 4ºC. It is assumed that alcohol fermentation microbes also decrease at the beginning of the post-fermentation stage so that the concentration of alcohol (ethanol) decreases.

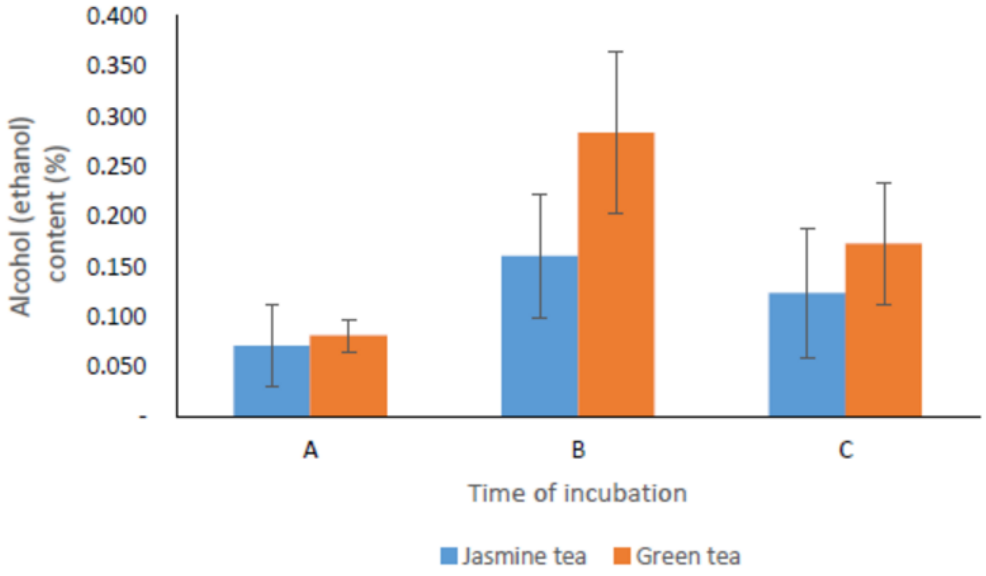

Figure 2. The alcohol (ethanol) content in jasmine and green tea kombucha. (A) Kombucha at fermentative stage (day 5 at 25ºC). (B) Kombucha at post-fermentative stage (day 7 at 4ºC). (C) Kombucha at post-fermentative stage (day 21 at 4ºC).

The total AAB of jasmine and green tea kombucha rose from day 5 of incubation to day 7 of the post-fermentative stage at at the 4ºC, then declined at the day 21 of the post-fermentative stage at the 4ºC (Table 1). It indicated that AAB could survive at the storage temperature for a week. According to Tan et al. (2020), the sucrose content in kombucha decreased ($p<0,05$) from the seventh d to the 21th d of the post-fermentation stage at storage temperature, causing its monomer to increase until the 14th day for fructose and 21th day for glucose. It was assumed that AAB used sucrose in kombucha as a nitrogen and carbon source to grow, then stopped when the sources were limited. The organic acids level of green tea kombucha rose from the fermentative stage to the post-fermentative stage ($p>0,05$) (Table 1). The increase of organic acid correlated with the decrease of alcohol (ethanol) at the onset of the post-fermentative stage. AAB used ethanol as a precursor for acetic acid fermentation to produce acetic acid (Mas et al. 2014). Lactic acid bacteria also produce the other organic acids through lactic acid fermentation (Michelz Beitel et al. 2017), so that organic acids accumulate at the onset of the post-fermentative stage. Kaewkod et al. (2019) reported that the acetic acid of kombucha inhibited the enteric bacteria growth at 1.72 g/L concentration. The pH level of kombucha jasmine declined from 4.24 to 4.05 and pH level of kombucha green tea declinde from 3.9 to 3.7 at the fermentative stage to the post-fermentative stage at storage temperature 4ºC. It was due to the accumulation of organic acids. Moreover, the acidic condition of kombucha can inhibit the contamination of pathogenic microbes. Kaewkod et al. (2019) reported that kombucha at the low pH value had antibacterial activity against enteric bacteria: *Escherichia coli*, *Shigella dysenteriae* DMST 1511, E. coli O157: H7 DMST 12743, *Vibrio cholerae*, and *Salmonella typhi* DMST 22842. The antimicrobial components were also thermostable. However, the neutralization of kombucha at pH 7.0 could not inhibit all of the tested enteric bacteria, neither did the unfermented kombucha. It is indicated that the activity of antibacterial of kombucha occurs when the pH level is low. Thus, the organic acids and the low pH of kombucha contribute to preventing the contamination of pathogenic microbes.

Table 1. Total AAB, organic acid concentration, and pH level of jasmine and green tea kombucha

Time of Incubation	Total AAB (CFU/mL)		Organic acid (%)		pH	
	Jasmine tea	**Green tea**	**Jasmine tea**	**Green tea**	**Jasmine tea**	**Green tea**
Five d incubation	18.167 ± 1.041	107.500 ± 2.500	0,245 ± 0,065	0,151 ± 0,033	4,24	3,9
Seven d of post- fermentative stage (4 °C)	112.500 ± 74.246	170.000 ± 26.514	0,189 ± 0,029	0,302 ± 0,033	4,1	3,74
21 d of post-fermentative stage (4 °C)	75.333 ± 6.526	92.667 ± 18.751	0,255 ± 0,050	0,434 ± 0,082	4,05	3,71

p>0,05 for Total AAB and organic acid

The ethanol level, AAB, and organic acid content of jasmine tea kombucha were lower than green tea kombucha. The organic acid concentration also had decreased at the seventh d before increased at the 21th day of the post-fermentative stage at the 4°C. The low concentration of organic acids in jasmine tea kombucha caused the pH level of jasmine tea higher than green tea kombucha. These were probably caused by the antifungal compound in jasmine tea that could inhibit yeast growth in kombucha. One of the examples is phenolic compounds in jasmine flowers (Sihite et al. 2018). Dang et al. (2014) reported that phenol could inhibit the growth of *S. cerevisiae*. Therefore, The ethanol level, AAB, and organic acid content of jasmine tea kombucha were low compared to green tea kombucha. In conclusion, jasmine and green tea kombucha at the fermentative stage (5 day at 25°C) and the post-fermentative stage (7 and 21 d, both at 4°C) did not produce more than 0.5 % ethanol without any significant differences in organic acids and AAB levels.

CONCLUSION

Jasmine and green tea kombucha at the fermentative stage (5 d at 25 °C) and the post-fermentative stage (7 and 21 d, both at 4°C) did not produce more than 0.5% ethanol without any significant differences in organic acids and AAB levels.

ACKNOWLEDGMENTS

This work was supported by Departement of Biotechnology, Universitas Muhammadiyah Bandung for the laboratory equipment.

REFERENCES

Adi IP (2018) Buku Panduan Membuat Teh Kombucha di Rumah. Wikikombucha, Indonesia

Arshad S, Nur F (2018) Alcohol â€“ halal or haram? what is alcohol and khamar? https://halal.upm.edu.my/article/alcohol__halal_or_haram_what_is_alcohol_and_khamar_-42087

BC Centre for Disease Control (2020) Food Safety Assessment of Kombucha Tea Recipe and Food Safety Plan. http://www.bccdc.ca/resource-gallery/Documents/Educational Materials/EH/FPS/Food/kombucha1.pdf. Accessed 14 Aug 2021

CDC (Centers for Disease Control and Prevention (2022) Alcohol Use and Your

Health. https://www.cdc.gov/alcohol/fact-sheets/alcohol-use.htm#:~:text=Long%2DTerm%20Health%20Risks,liver%20disease%2C%20and%20digestive%20problems.&text=Cancer%20of%20the%20breast%2C%20mouth,liver%2C%20colon%2C%20and%20rectum.

Crawford RA, Pavitt GD (2019) Translational regulation in response to stress in *Saccharomyces cerevisiae*. Yeast 36:5–21. doi: 10.1002/yea.3349

Dang D, Wang Z, Thygesen A, Wang C, Zhou W, Xing J, Lin W (2014) Bio-oil Treated by Cultivation of Saccharomyces cerevisiae (QH01). Bioresources 9. doi: 10.15376/biores.9.2.2727-2738

Gullo M, Caggia C, De Vero L, Giudici P (2006) Characterization of acetic acid bacteria in "traditional balsamic vinegar." Int J Food Microbiol 106:209–212. doi: 10.1016/j.ijfoodmicro.2005.06.024

Heryani (2019) Memahami fatwa mui tentang kadar etanol pada produk makanan dan minuman. http://www.halalmui.org/mui14/index.php/main/detil_page/48/27571. Accessed 14 Aug 2021

HMC, Halal Monitoring Committee (2024) Why is it so important for a Muslim to strictly consume Halal? https://halalhmc.org/resources/importance-of-halal/

Ihsani N, Hernahadini N, Fauzi M (2021) The variation of ethanol concentration and kombucha characterization on several incubation periods. J Phys Conf Ser 1764:012008. doi: 10.1088/1742-6596/1764/1/012008

Jakubczyk K, Kałduńska J, Kochman J, Janda K (2020) Chemical Profile and Antioxidant Activity of the Kombucha Beverage Derived from White, Green, Black and Red Tea. Antioxidants 9:447. doi: 10.3390/antiox9050447

Kaewkod T, Bovonsombut S, Tragoolpua Y (2019) Efficacy of Kombucha Obtained from Green, Oolong, and Black Teas on Inhibition of Pathogenic Bacteria, Antioxidation, and Toxicity on Colorectal Cancer Cell Line. Microorganisms 7:700. doi: 10.3390/microorganisms7120700

Laureys D, Britton SJ, De Clippeleer J (2020) Kombucha Tea Fermentation: A Review. Journal of the American Society of Brewing Chemists 78:165–174. doi: 10.1080/03610470.2020.1734150

Liszkowska W, Berlowska J (2021) Yeast Fermentation at Low Temperatures: Adaptation to Changing Environmental Conditions and Formation of Volatile Compounds. Molecules 26:1035. doi: 10.3390/molecules26041035

Lobo *, Dias RO, Shenoy FO (2017) Kombucha for healthy living: Evaluation of antioxidant potential and bioactive compounds. 24(2):541-546

López-Malo M, Querol A, Guillamon JM (2013) Metabolomic Comparison of Saccharomyces cerevisiae and the Cryotolerant Species S. bayanus var. uvarum and S. kudriavzevii during Wine Fermentation at Low Temperature. PLoS One 8:e60135. doi: 10.1371/journal.pone.0060135

Mas A, Torija MJ, García-Parrilla M del C, Troncoso AM (2014) Acetic Acid Bacteria and the Production and Quality of Wine Vinegar. The Scientific World Journal 2014:1–6. doi: 10.1155/2014/394671

Michelz Beitel S, Fontes Coelho L, Sass DC, Contiero J (2017) Environmentally Friendly Production of D(−) Lactic Acid by *Sporolactobacillus nakayamae* : Investigation of Fermentation Parameters and Fed-Batch Strategies. Int J Microbiol 2017:1–11. doi: 10.1155/2017/4851612

Neffe-Skocińska K, Sionek B, Ścibisz I, Kołożyn-Krajewska D (2017) Contenido de ácido y efectos de las condiciones de fermentación en las propiedades fisicoquímicas, microbiológicas y sensoriales de bebidas de té de Kombucha. CYTA - Journal of Food 15:601–607. doi: 10.1080/19476337.2017.1321588

Ross P, Mayer R, Benziman M (1991) Cellulose biosynthesis and function in bacteria. Microbiol Rev 55:35–58. doi: 10.1128/mr.55.1.35-58.1991

Saxena Im, Brown Rm (2005) Cellulose Biosynthesis: Current Views and Evolving Concepts. Ann Bot 96:9–21. doi: 10.1093/aob/mci155

Sihite NW, Rusmarilin H, Suryanto D, Sihombing DR (2018) Utilization of jasmine flower extract as antimicrobial in tempeh sausage. IOP Conf Ser Earth Environ Sci 205:012037. doi:10.1088/1755-1315/205/1/012037

Tan WC, Muhialdin BJ, Meor Hussin AS (2020) Influence of Storage Conditions on the Quality, Metabolites, and Biological Activity of Soursop (Annona muricata. L.) Kombucha. Front Microbiol 11. doi: 10.3389/fmicb.2020.603481

TTB A and TT and TB (2019) Kombucha. https//www.ttb.gov/kombucha. Accessed 14 Aug 2021

Villarreal-Soto SA, Beaufort S, Bouajila J, Souchard J-P, Taillandier P (2018) Understanding Kombucha Tea Fermentation: A Review. J Food Sci 83:580–588. doi: 10.1111/1750-3841.14068