Isolation and Characterization of Microorganisms From Scoby (Symbiotic Culture of Bacteria and Yeast) During Kombucha Fermentation

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Abstract

Kombucha is made by fermenting tea with sugar solution using SCOBY (symbiotic culture of bacteria and yeast). Kombucha fermentation is divided into several stages, such as the conversion of sugar into ethanol, the conversion of ethanol into acetic acid, and the conversion of acetic acid into carbon dioxide. Thus, several types of unique microorganisms must be involved during kombucha fermentation. Our previous research has reported that culturable microorganisms from kombucha drink (liquid phase) were all bacteria. Furthermore, in this research we investigated culturable microorganisms from the SCOBY itself. During kombucha fermentation, SCOBY sheets were cut (about 1×1 cm) each day using a sterile knife. SCOBY slice was enriched in Potato Dextrose Broth and incubated at 37 ℃ for 24 hours. The enriched culture was inoculated into Plate Count Agar and incubated at 37 ℃ for 24 hours. Four different colonies, named isolate (a), (b), (c), (d), were collected during 14 days of kombucha fermentation. The suspected colonies of bacteria were cultivated in Nutrient Agar, while the suspected colonies of mold or yeast were cultivated in Potato Dextrose Agar. The characterization results suggested that isolate (a) has close characteristic to *Acetobacter* genus (gram negative, short rod, does not produce endospore), meanwhile isolate (b) is gram negative, long rod, and produces endospore. Isolate (c) was suspected as mold, and isolate (d) was identified as yeast.

Keywords: Bacteria; Fermentation; Kombucha; SCOBY; Yeast

Abstrak

Kombucha merupakan produk minuman tradisional hasil fermentasi larutan teh dan gula dengan menggunakan starter kultur kombucha atau lebih dikenal dengan kultur SCOBY *(Symbiotic Culture of Bacteria and Yeast)*. Waktu fermentasi kombucha terbagi menjadi beberapa tahapan, yaitu konversi gula menjadi etanol, konversi etanol menjadi asam asetat, dan konversi sebagian asam asetat menjadi karbon dioksida. Setiap tahapan fermentasi melibatkan mikroorganisme yang berbeda. Penelitian sebelumnya didapatkan bahwa semua mikroorganisme yang terdapat pada fasa cair kombucha adalah bakteri. Selanjutnya, dalam penelitian ini dilakukan penelitian lanjutan yaitu isolasi mikroorganisme pada lembaran SCOBY. Selama fermentasi kombucha, lembaran SCOBY dipotong (sekitar 1x1 cm) setiap hari menggunakan pisau steril. Potongan SCOBY diperkaya dengan Potato Dextrose Broth dan diinkubasi pada suhu 37 ℃ selama 24 jam. Kultur yang diperkaya diinokulasi ke dalam Plate Count Agar dan diinkubasi pada suhu 37 ℃ selama 24 jam. Empat koloni berbeda yang diberi nama isolat (a), (b), (c), dan (d) didapatkan selama 14 hari fermentasi kombucha. Koloni yang diduga bakteri ditumbuhkan dalam Nutrient Agar, sedangkan koloni yang diduga sebagai kapang atau khamir ditumbuhkan dalam Potato Dextrose Agar. Hasil karakterisasi menunjukkan bahwa isolat (a) memiliki sifat yang mirip dengan genus *Acetobacter* (gram negatif, batang pendek, dan tidak menghasilkan endospora), isolat (b) bersifat gram negatif, batang panjang, dan menghasilkan endospora. Isolat (c) diduga sebagai kapang, dan isolat (d) diidentifikasi sebagai khamir.

Kata Kunci: Bakteri; Fermentasi; Kombucha; SCOBY; Yeast

INTRODUCTION

Kombucha is made by fermenting tea with sugar solution using SCOBY (Symbiotic Culture of Bacteria and Yeast) (Aditiwati, 2003). SCOBY is white gel sheet wrapped by a membrane that consists of bacteria (*Acetobacter xylinum*, *Acetobacter ketogenum* and *Bacterium gluconicum*) and yeast (*Candida albicans*, *Saccharomyces*, *Pichia fermentants*, *Brettanomyces*,

and Zygosaccharomyces) (Anugrah, 2005). Prolonged sugar fermentation leads to carbon dioxide production, which gives soda sensation in kombucha drink alongside with tea flavor and aroma.

Kombucha fermentation time ranges from 8-12 days at 18-20ºC, while at higher temperatures the fermentation takes a shorter time. Kombucha fermentation is divided into several stages, such as the conversion of sugar into ethanol, the conversion of ethanol into acetic acid, and the conversion of acetic acid into carbon dioxide. Different conditions in each fermentation phase will affect the variety of microorganisms. Thus, several types of unique microorganism (range from yeast to bacteria) must be involved during kombucha fermentation.

Recent studies had reported that the most dominant yeast in SCOBY was *Candida* sp., meanwhile in the liquid phase the dominance was shifted from *Candida* sp. to *Lachancea* sp. during kombucha fermentation. The most dominant bacteria in kombucha was *Komagateibacter*, a member of *Acetobacteraceae* family (Chakravorty et al., 2016). Another metagenomic approach showed that bacteria *Komagataeibacter rhaeticus* and yeast *Brettanomyces bruxellensis* were the most common microorganism in 23 samples of kombucha across the United States (Landis, et al.,2022)

Our previous research, focused on cultivation approach, has also reported that culturable microorganisms from kombucha drink (liquid phase) were all bacteria. Four out of six bacteria isolates showed close characteristic to *Acetobacter* genus (gram negative, short rod, motile, and does not produce endospore) (Permana, et al., 2021). However, in that study, no yeast was found in liquid phase. So that in this research we investigated culturable microorganisms from the SCOBY (solid phase which is the cellulose sheets in kombucha).

MATERIALS AND METHODS

Materials

The materials used in this study were green tea, sugar, water, aquades, SCOBY, Potato Dextrose Broth (PDB), Plate Count Agar (PCA), Potato Dextrose Agar (PDA), Nutrient Agar (NA), safranin, crystal violet, malachite green, 95% alcohol, lugol solution, immersion oil, sodium acetate (CH_3COONa), acetic acid (CH_3COOH), buffer solutions pH 7 and pH 9.

Kombucha Production and pH Measurement

Kombucha is made based on Naland procedure (2004) with some modifications. Two pieces of green tea bag were dissolved in 800 mL boiling water for 15 minutes. Eighty grams (10% *w*/*v*) of granulated sugar was added into the tea solution. After its temperature reached 20-25ºC, the solution was poured into a sterilized glass container and covered with a clean cloth. A piece of SCOBY and 8 mL (1% *v*/*v*) kombucha starter from the previous batch were added to the solution. The container was kept out from the light and stored at 23-27 ºC for 14 days. The acidity of tea solution was measured each day during kombucha fermentation, so profile of pH during fermentation process could be obtained.

Microorganism Isolation

During kombucha fermentation, SCOBY sheets were cut (about 1×1 cm) each day using a sterile knife. SCOBY slice was enriched in PDB and incubated at 37 ℃ for 24 hours. The enriched culture was inoculated into PCA and incubated at 37 ℃ for 24 hours. The suspected colonies of bacteria were cultivated in NA media with zigzag angle, while the suspected colonies of mold or yeast were cultivated in PDA using streak plate technique.

Morphology Identification of Microorganism

The morphology of bacteria and yeast was identified by simple staining. Further identification of bacteria was carried out by Gram staining and endospore staining. The preparate glasses of simple, Gram, and endospore staining were observed using a microscope with 100× magnification of the objective lens. The morphology of mold was observed directly using a microscope with 4× magnification of the objective lens.

RESULTS AND DISCUSSION

Profile of pH during Kombucha Fermentation Process

Kombucha is a fermented product that produces acid during the process. As an indicator that the fermentation process has occurred is a change of pH value. During fermentation, the pH value of kombucha in this study was decreased (Figure 1) from 4.1 to 2.8 in the first 24 hours. On the next day until the 14th day of fermentation, the pH tends to be stable at 2.6.

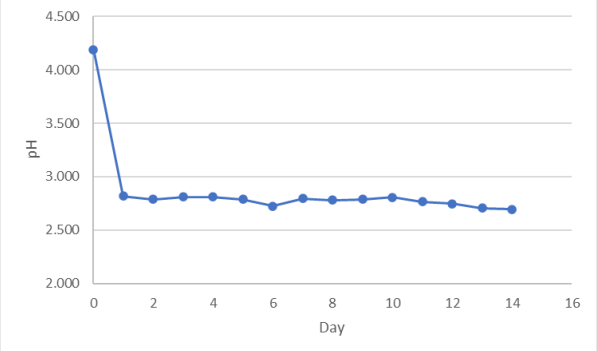

Figure 1. Kombucha pH Profile During the Fermentation Process

During kombucha fermentation, sugar provides nutrition for microorganisms. The yeast has an important role in sugar fermentation into ethanol, which is then broken down by bacteria into organic acids such as glucuronic acid, acetic acid, and lactic

acid. The concentration of organic acid increased during the fermentation process, causing a rapid decrease in pH value. *Acetobacteraceae* family plays

Table 1. Colonies Morphology of SCOBY Isolate

Colonies	Shape	Texture	Color	Surface	Elevation	Edge
(a)	Circular	Smooth	Milky White	Dull	Raised	Entire
(b)	Irregular	Smooth	Transparent White	Weak glistening	Crateriform	Undulate
(c)	Filamentous	Wrinkled	Cream	Establish zoning	Hyphae	Filiform
(d)	Circular	Smooth	Cream	Glistening	Convex	Entire

important role to the pH profile of kombucha through their metabolic activity. The decrease in pH value during fermentation gives suitable environment for the microorganism in kombucha. Acid production occurs continuously, but the pH tends to be stable at 2.6 after 24 hours of fermentation until the 14th day of fermentation. This is because at the same time, microorganisms convert acid into carbon dioxide which gives the sensation of a fizzy taste in kombucha.

Microorganism Isolate and Its Purification

Isolation of microorganisms from SCOBY was carried out using PCA. PCA is commonly used to count the total number of microorganisms, therefore bacteria, yeast, and mold could growth in PCA. Before being cultivated in PCA, SCOBY slice was enriched in PDB to provide initial nutrients to support the growth of microorganisms. Four different colonies were collected during 14 days of kombucha fermentation. Those colonies showed different characteristics from each other (Figure 2). The morphology identification of those colonies was carried out with characteristics observation such as shape, texture, color, surface, elevation, and the edge of the colony (Table 1).

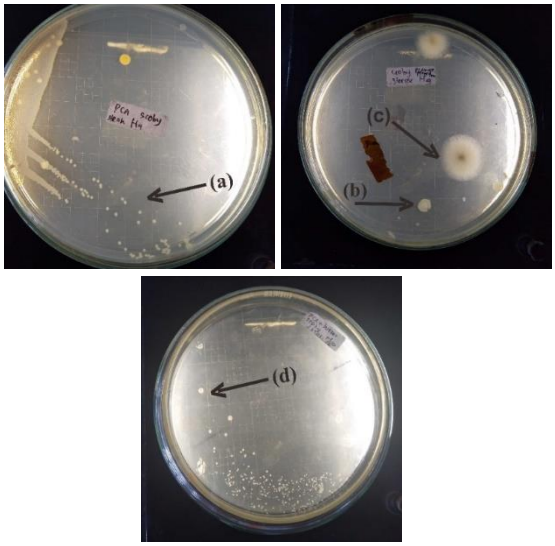

Figure 2. Morphology of Isolated Colonies (a), (b), (c), and (d)

Based on morphological characteristics, it was assumed that isolate (a) and (b) are bacteria, isolate (c) is mold, and isolate (d) is yeast. Isolate (a) and (b) were purified by cultivation in NA media, meanwhile isolate (c) and (d) were purified by cultivation in PDA media (Figure 3).

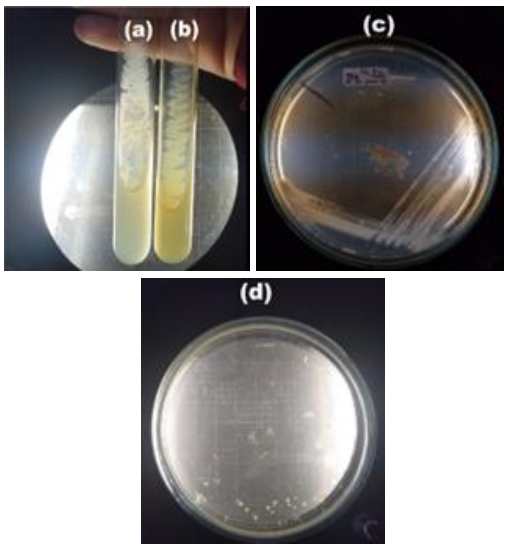

Figure 3. Purification Results of Bacteria (a and b) on NA Media, Mold (c) and Yeast (d) on PDA Media

Microorganism Morphology

Simple staining provides easier observation of the cell shape and the arrangement of microorganisms. Simple staining results showed that isolate (a) had short rod shape, isolate (b) had long rod shape, and isolate (d) had elliptical and round shape (Figure 4).

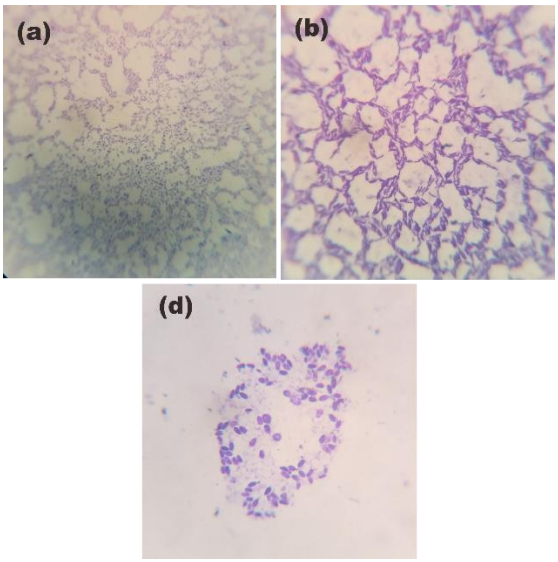

Figure 4. Simple Staining of Isolate (a), (b), and (d)

Isolate (d) had similarities with yeast. Simple staining results showed the cells had round and elliptical/oval shapes, a vegetative reproductive system with multilateral budding, did not form pseudo hyphae and produced ascospores (Figure 5).

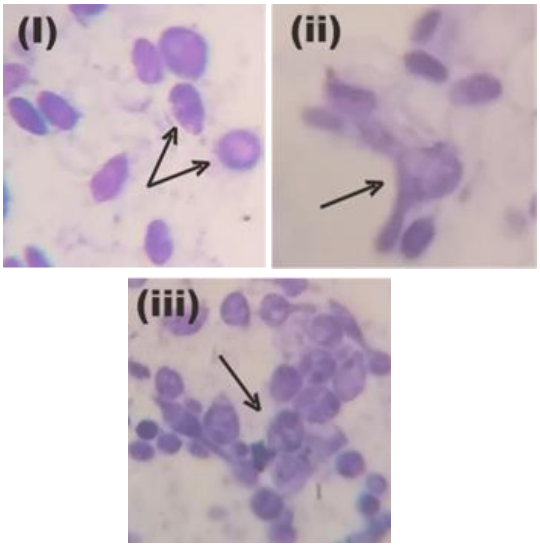

Figure 5. Simple Staining of Yeast (i) Cell Shape, (ii) Budding, and (iii) Ascospore

Gram and endospore staining were carried out on isolate (a) and (b). Gram staining results showed that the isolate (a) and (b) were Gram negative bacteria (Figure 6). Endospore staining results showed that the isolate (a) did not produce spores, while isolate (b) produced endospores marked in green (Figure 7).

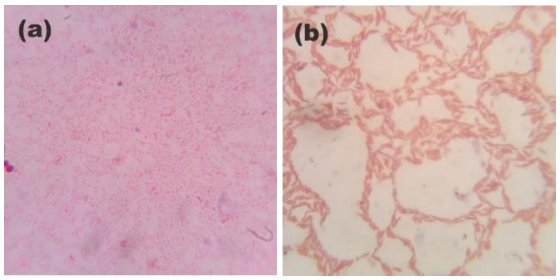

Figure 6. Gram Staining of Isolate (a) and (b)

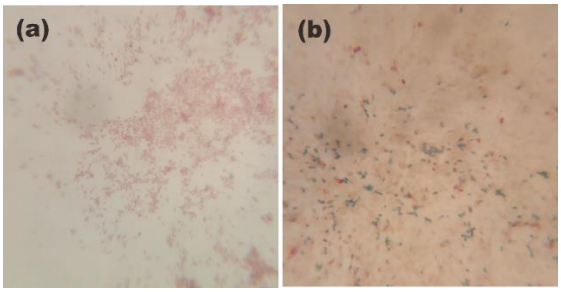

Figure 7. Endospore Staining of Isolate (a) and (b)

Isolate (c) was suspected to be mold because it has hyphae that form mycelium. Microscopic observations of molds included the sporangium shape, the hyphae shape, and the pigmentation of the hyphae. Isolate (c) had semi-spherical to round sporangium, with the hyphae had rhizoid (fibrous) shape and pigmented dark (Figure 8).

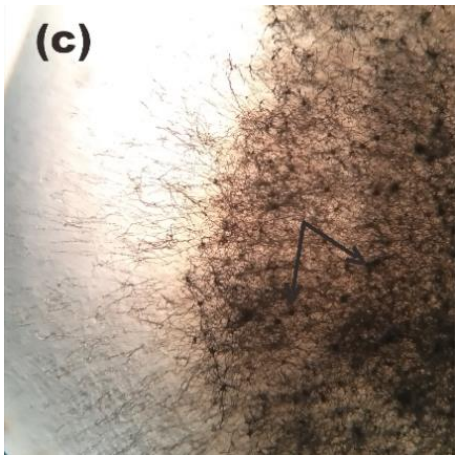

Figure 8. Morphology of Isolate (c)

Based on Bargey's Manual of Determinative Bacteriology (Holt et.al., 2004), the characteristics found in isolate (a) are similar to *Acetobacter* genus. The characteristics of *Acetobacter* is elliptical to rod-shaped, straight, or slender and some strains can be round, elongated, bent, or filamentous. *Acetobacter* cells do not form endospores and belong to Gram-negative (-) bacteria. Meanwhile, isolate (b) has a long rod shape, belong to gram-negative bacteria and

produces endospores. The results showed that isolate (b) has different characteristics from *Acetobacter* genus. Isolate (c) was suspected as molds because it has hyphae that form mycelium. According to Kurtzman (2011), isolate (d) has similarities with yeast. The characteristics of isolate (d) are similar to *Saccharomyces* genus, such as cell shape (round, short ovals or ovals), multilateral budding, produces ascospores, and does not form pseudohyphae and true hyphae (Nurhariyati, 2004).

The microorganisms isolated from SCOBY are in accordance with the statement from Greenwalt (1998), a mixed culture of microorganisms in kombucha consists of yeast and acetic acid bacteria. The main bacteria come from the genus *Acetobacter*, in particular (*Acetobacter xylinum, Acetobacter xylinoides, and Bacterium gluconium*) and yeast components (*Saccharomyces pombe, Saccharomyces ludwigii, Saccharomyces cerevisiae, Pichia fermentant*) (Wong, 2001).

CONCLUSION

Culturable microorganisms from SCOBY obtained in this research was vary from bacteria, mold, and yeast. Two of the isolates have close characteristics to the main microorganism in kombucha, which is *Acetobacter* (bacteria) and *Saccharomyces* (yeast). Those microorganisms were present during the kombucha fermentation, along with the growth of SCOBY.

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