Effect of *Dekkera* and *Komagataeibacter* ratio on kombucha quality in different percent of sugar

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Abstract

Different ratio of yeast (*Dekkera bruxellensis*) to bacteria (*Komagataeibacter intermedius*) on kombucha fermentation at different sugar levels were investigated. The results indicated that at the ratio of 1:1, the growth and metabolic activity of both microorganisms were good at all levels of sugar but the fermentation time were not terminated in 6 days. Kombucha quality at day 6 had alcohol content of 2.25-4.83% and acetic acid content of 2.46-3.87%. Changing of *Dekkera bruxellensis* to *Komagataeibacter intermedius* ratio as 1:2 made the fermentation completed in 6 days. At all sugar levels for fermentation the final products had acetic acid content of 2.4-4.3 since day 2 of fermentation. It was found that as the fermentation time increased acetic acid content tended to stable at 3% when using sugar content of 10-14% and stable at 4-5% when using sugar content of 16-20%. While the ratio of *Dekkera bruxellensis* to *Komagataeibacter intermedius* as 2:1, the fermentation time did not finish in 6 days. At all levels of sugar in this study produced 4% alcohol at day 4 of fermentation and at sugar content of 10-16% alcohol and acetic acid content were 4.10-4.22% and 3.00-3.25% respectively.

Keywords: *Dekkera , Komagataeibacter , Kombucha, acetic acid, biofilm*

1. Introduction

Kombucha is a healthy fermented tea beverage produced by fermentation of tea leaves and sucrose with a symbiotic culture of yeast and acetic acid bacteria. At the first stage of fermentation, invertase from yeast digest sucrose to glucose and fructose and convert these monosaccharides into ethanol at a low level that enough to activate acetic acid bacteria growth, which oxidizes alcohol to acetic acid in the second stage of fermentation (Jayabalan *et al*., 2007). However, the sour taste of organic acid in Kombucha resulted from the metabolism of both yeast and acetic acid bacteria. Besides acetic acid, gluconic acid and a cellulosic biofilm are formed as metabolites at the same time as shown in Figure1 (May et al. 2019). Moreover, many reports indicated that there were other benefit metabolites for the consumer in Kombucha such as glucuronic acid, oxalic acid, butyric acid, lactic acid, tartaric acid), malic acid) as well as citric acid in low level including glucuronidase enzyme that has antimicrobial activity and anti toxicity from chemotherapy (Srinivasan *et al.*, 1997). Also, there was DSL or D-saccharic acid-1,4-lactone, a substance helping the liver to detoxify toxin and carcinogen (Martinez et al., 2018; Zhiwet *et al.*,2010). Kombucha is a popular beverage nowadays. The value of the global kombucha market is estimated to be USD 1.5 billion in 2018. From 2014 to 2018, the market grew at a compound annual growth rate (CAGR) of 23%. The market is forecasted to continue its growth, reaching between USD 3.5 to 5 billion by 2025 (Kim & Ad Hikari, 2020). It is a non-thermal pasteurized fermented beverage that remains the activity of enzymes. It tastes slightly sour with a fresh sparkling taste from carbonate. Many types of tea can be used as raw materials for Kombucha production since each tea has different color and taste depend on the composition of tea leaves, processing and starter culture which is a mixed culture called Tea fungus mat or SCOBY (symbiotic culture of bacteria and yeast). After fermentation is complete, this fermented beverage will contain low alcohol content as 0.5-1% and taste like sparkling vinegar cider. It also contains vitamin B and other nutrients that are good for health and provided the same benefits of probiotics in supplementary foods. According to commercial Kombucha sold in the United States, more than 300 brands contain alcohol. In general, the alcohol content in this beverage is 1-2%; however, Kombucha that contains lower 0.5% alcohol is classified as a non-alcoholic beverage (Kumar & Joshi, 2016). The recommended intake of Kombucha was 100-300

ml (Frank, 1991). The screening and identification report for SCOBY indicated that the culture composed of many yeasts strains both saccharomyces and non- saccharomyces yeast, including aerobic and anaerobic acid bacteria. The species can be classified into six groups: (1) Saccharomyces yeast such as *Saccharomyces cerevisiae and Saccharomyces bisporus*, (2) Non- *Saccharomyces* yeast such as *Saccharomycoides ludwigii, Zygosaccharomyces bailii, Zygosaccharomyces kombuchaensis, Z. rouxii,Candida krusei, Kloekera/Hanseniaspora apiculata, Torulopsis, Torulaspora delbrueckii , Pichia, Brettanomyces/ Dekkera bruxellensis, Schizosaccharomyces pombe and Kluyveromyces.* For bacteria, there are acetic and lactic acid bacteria including gluconic acid bacteria and biofilm-producing bacteria, (3) Acetic acid-producing bacteria such as *Acetobacter aceti, Acetobacter xylinoides, Acetobacter pasteurianus* and *Gluconobacter oxydans*, (4) Lactic acid-producing bacteria such as *Lactobacillus sp., Lactococcus sp.*, and *Bifidobacterium sp*, (5) Gluconic acid-producing bacteria such as *Komagataeibacter xylinus*, and *Bacterium gluconicum*, and (6) Cellulose producing bacteria which produce cellulosic biofilm or uridine diphosphate-glucose (UDPGlc) such as *Komagataeibacter xylinum, Acetobacter spp.*,and *Gluconobacter oxydans* (Villarreal-Soto *et al*., 2018; Chakravorty *et al*., 2016). In general, Kombucha fermentation process after acid content was up to 3% it affected the activity of starter culture, including the taste of the final product. Therefore, fermentation time should not be too long (Greenwalt *et al*., 2000). Many researchers used different yeast and bacterial strains that differ from this study for Kombucha production. Most reported yeast strains were *Brettanomyces, Zygosaccharomyces* and *Saccharomyces* (Liu *et al.*,1996; Roussin,1996; Mayser *et al.*,1995), *Pichia* and *Zygosaccharomyces* (Hesseltine, 1965) *Torulopsis, Mycotorula, Schizosaccharomyces, Torula, Mycoderma and Candida* (Jankovic & Stojanovic, 1994). Most reported bacterial strains were *Acetobacter xylinum* (Fontana *et al*.,1991; Jankovic & Stojanovic,1994; Liu *et al.*,1996; Mayser *et al.*,1995; Roussin,1996; Sievers *et al.*,1995), *Gluconacetobacter xylinus* and *Komagataeibaccter xylinus* (Yamada *et al.*,2012). Nowadays, there are commercial Kombucha starter sold as Scoby biofilm and mixed culture. Black tea is commonly used as a raw material in combination with sugar. The process starts by adjusting sugar concentration to 5-15% then adding starter from the former fermentation which is the component of Scoby agar sheet and fermented liquid by using 2.5-3% (w/v) Scoby biofilm in combination with 10-20% (v/v) fermented liquid. The fermentation time was about 10-21 days (Gaggia *et al.*,2019; Kumar&Joshi, 2016; Jayabalan *et al.*,2014; Greenwalt *et al.*,2000). Muhialdin *et al*. (2019) reported the using of mixed starter culture as Scoby biofilm with an inoculum size of 3% to produce Kombucha from black tea at 10% sugar concentration and found that fermentation time was 14 days. Zhiwei *et al.* (2010) found that the kombucha made from black tea fermented at 10% sugar concentration using mixed culture had the maximum growth of acetic acid bacteria at day 4 of fermentation as kombucha as 7.87 logs CFU/ml with a maximum acid content of 3.406%. While Kombucha fermentation from black tea at 10% sugar concentration by Velicanski *et al.* (2007) showed that using inoculum size of 2.5% resulting in suitable and acceptable acid content within 7-10 days of fermentation. According to pure starter culture for Kombucha fermentation from black tea at 10% sugar concentration by Mankasetkit & Bovornsombat (2017), it was found that growth of acetic acid bacteria and yeast were maximum at day 6 of fermentation as 6.86 and 7.08 log CFU/ml respectively. The maximum alcohol content was 4.1% at day 9 of fermentation, and acetic acid content increased with time and was maximum at 1.808% concentration.

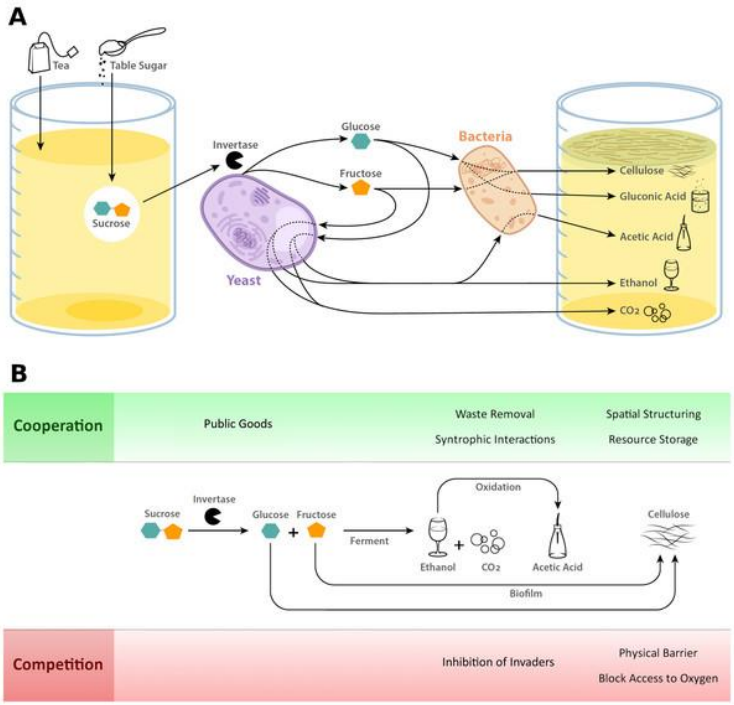

Figure 1 Kombucha metabolism and microbial interactions (May *et al.*, 2019)

However, The effect of different sugar concentrations on fermentation times is not clear. The ratio of microorganism in Scoby for good fermentation has not been studied. The aim of this research needs to study the effect of yeast and specific pure bacterial strain for Kombucha beverage by using *Dekkera bruxellensis* as yeast strain and *Komagataeibacter intermedius* as bacterial strain. The symbiotic fermentation of the two microorganisms produces alcohol, carbon dioxide, and acetic acid. Greenwalt *et al.* (2000) revealed that fermentation of Kombucha from black tea could be done in 7-10 days by using inoculum size of mixed culture at 2.5% and sugar concentration at 10%. The result was agreed with our preliminary study (Puangwerakul,2020) which processed Kombucha from black tea with a pure strain of *D. bruxellensis* and *K. intermedius* at the ratio of 1:1 by varying different sugar concentration between 10-20% and varying inoculum size between 2-10% for seven days of fermentation time. It was found that acetic acid was produced at the same suitable concentration of 3% but the thickness of biofilm were formed as shown in Figure 2 and 3. So, the primary conclusion is that it is not necessary to use starter culture up to 10% because using of 2% starter culture can produce suitable acetic acid content of 3% which can inhibit the growth of contaminated microorganism both spoilage and pathogenic type (Greenwalt *et al.*, 2000). This process can be conveyed to the community or entrepreneur for commercial production.

Figure 2 Kombucha from black tea fermentation at day 7 using the ratio of *Dekkera*: *Komagataeibacter* as 1:1, inoculum size of 2% at sugar concentrations of 10, 12, 14, 16, 18, and 20% (from left to right) (Puangwerakul, 2020).

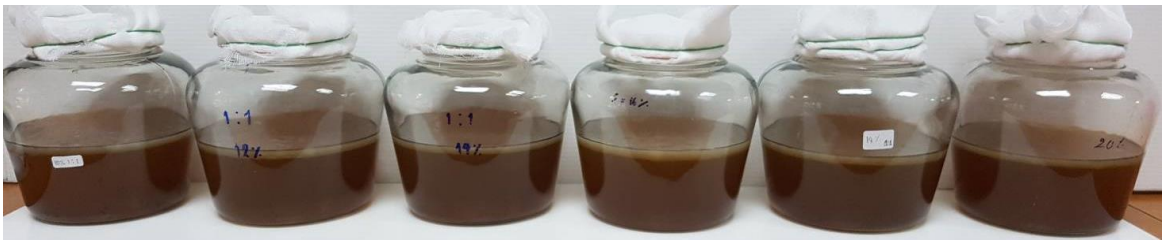

Figure 3 Kombucha from black tea fermentation at day 7 using the ratio of *Dekkera*: *Komagataeibacter* as 1:1, inoculum size of 10% at sugar concentrations of 10, 12, 14, 16, 18, and 20% (from left to right) (Puangwerakul, 2020).

The data from the preliminary study showed that preparation and using pure yeast strain *Dekkera bruxellensis* and pure bacterial strain *Komagataeibacter intermedius* for production of Kombucha instead of mixed culture in Scoby had better fermentation outcomes not only reduced contamination during fermentation but also could control and fix the fermentation time. However, it is not clear that what is the optimum sugar concentration and the suitable ratio of the yeast and bacterial strain for good Kombucha fermentation which can apply to commercial production.

2. Objectives

To study the effect of different ratio of *Dekkera bruxellensis*: *Komagataeibacter intermedius* and different sugar concentration on Kombucha quality using 2% starter culture

3. Materials and Methods

3.1 Preparation of starter culyure for fermentation

Inoculum of *Dekkera bruxellensis* was prepared by adding one loopful of the culture in YM broth then shaking at 190 rpm at room temperature for 24 hours. After that, counted the number of cells and diluted the concentration of cell suspension with 0.1% peptone to obtain the initial cell suspension of $1.5x10^8$ cell/ml.

In order to prepare *Komagataeibacter intermedius*, one loopful of the culture was added to GYE broth, then shaking at 190 rpm at room temperature for 48 hours. After that, counted the number of cells and diluted the concentration of cell suspension with 0.1% peptone to obtain the initial cell suspension of $1.5x10^8$ cell/ml.

For varying the different ratio of a starter culture of *Dekkera bruxellensis*:*Komagataeibacter intermedius* to obtain an inoculum size of 2% of 250 ml fermentation medium, the volume calculations were done as follow:

ratio 1:1; mixed 2.50 ml yeast suspension with 2.50 ml bacterial suspension
ratio 1:2; mixed 1.67 ml yeast suspension with 3.33 ml bacterial suspension
ratio 2:1; mixed 3.33 ml yeast suspension with 1.67 ml bacterial suspension

3.2 Preparation of tea solution

Ten grams of black tea leaves were added in 1 litre boiled water and continued to boil for 5 minutes, then removed out the leaves by filtration. Sugar at different concentrations (10% (25 g), 12% (30 g), 14% (35 g), 16% (40 g), 18% (45 g), and 20% (50 g)) were added and dissolved by stirring, tea solutions were boiled again for 5 minutes. After boiling, these solutions were kept in sterilized glass bottles (250 ml/ bottle), the lids were closed, and the bottles were cooled down to room temperature. The starter of yeast and bacteria were added as the volume calculated from 3.1 into the cooled down black tea, and the glass bottles were closed with sterilized straining cloth, then they were incubated at room temperature for six days.

3.3 Quality analysis during fermentation

Samples were taken out at day 2, 4, and 6 to analyze °Brix by Hand Refractometer, alcohol by Vinometer, and total acid as acetic acid by titration method with 0.1N NaOH using phenolphthalein as an indicator and biofilm thickness as mm by measuring tape.

3.4 Statistical analysis

The treatment and analysis in this study were done in three replications. The data were presented as mean, and their differences were compared using DMRT (Duncan's New Multiple Range Test) by SPSS for windows version 12.0.

4. Results and Discussion

The data showed that using the ratio of *Dekkera bruxellensis* to *Komagataeibacter intermedius* as 1:1 at any levels of sugar concentration. The results were in the same direction. The °Brix value or total soluble solid decreased while alcohol and acetic acid contents continuously increased until day 6 of fermentation. The acetic acid content was found between 2.46-3.87%, which similar to 3% acetic acid of standard Kombucha. It could be explained that symbiotic work of yeast and bacteria as the ratio of yeast: bacteria as 1:1 was good, so they could change sugar to alcohol and then to acetic acid respectively. As the same time, the cellulosic network was formed (5.0-6.0 mm) due to bacterial growth. Therefore, the yeast: bacterial ratio best suited for Kombucha fermentation was 1:1 because growth and fermentation activity of both microorganisms were good at all levels of sugar concentration between 10-20%. The fermentation time did not finish in 6 days, but the kombucha quality contained suitable acid content for consuming, as shown in Table 1.

When using *Dekkera bruxellensis*: *Komagataeibacter intermedius* as 1:2, it was found that whether using sugar concentrations, the results were in the same direction. These were a decreasing of °Brix value or total soluble solid, which closed to the value when using the ratio of yeast: bacterial of 1:1 and accompanied with continuous increasing of alcohol until day 6 of fermentation. However, alcohol contents at every stage of fermentation were low and similar to 2.20-2.75% at all levels of sugar concentration which were lower than treatment using the ratio of yeast: bacterial of 1:1. It could be explained that the higher of bacterial ratio affected to higher changing of alcohol to acetic acid. Besides, the data also showed that at every concentration of sugar used, 2.4-4.36% acetic acid contents were obtained since day 2 of fermentation. However, it was noticed that when fermentation time increased acetic acid contents tended to stable at 3% at various sugar concentrations of 10-14% and when using sugar concentration of 16-20% acetic acid contents were 4-5%. Therefore, Kombucha fermentation when using *Dekkera bruxellensis*: *Komagataeibacter intermedius* as 1:2 the suitable sugar concentrations were 10-14% because fermentation activity and growth of both microorganisms were good and complete fermentation occurred in 6 days as shown in Table 1.

For the ratio of *Dekkera bruxellensis*: *Komagataeibacter intermedius* as 2:1, the results also indicated that there were a decreasing of °Brix or total soluble solid together with a continuous increase of

alcohol and acetic acid contents until day 6 of fermentation. Acetic acid and alcohol contents were between 3.0-5.62% and 4.10-7.00%, respectively. The increase in yeast proportion resulted in the increase in alcohol production to 4% at day 4 of fermentation. However, it was noticed that at 18-20% sugar concentration although the fermentation did not complete, but 6.75-7.0% alcohol and 5.60-5.62% acetic acid were produced which affected the bacterial growth and resulted in low biofilm thickness (an indirect indicator). At sugar concentration of 18% and 20%, the thickness of biofilm were only 1.15 and 0.73 mm because the higher alcohol is toxic to bacterial cells. Moreover, production of acetic acid at 5% or pH 2.5 (data not shown) impacted on inhibition of glucosyltransferase and phosphodiesterase which related to cellulose synthesis of *Komagataeibacter intermedius*, one of *Acetobacter spp*. The Acetobacter work well at pH 3.0-7.0 (Lannino *et al*,1988;Sievers *et al*.,1995; Chen&Liu, 2000; Zhang, Zhang & Xin,2011). Therefore, Kombucha fermentation using *Dekkera bruxellensis*: *Komagataeibacter intermedius* ratio of 2:1 at sugar concentration of 10-16% were suitable for good fermentation activity and growth of both microorganisms. Although the fermentation did not complete in 6 days, but Kombucha with 3% acetic acid content which suitable for consuming was obtained.

Table 1 Changing of °Brix, alcohol and acetic acid content including biofilm thickness during Kombucha fermentation at different *Dekkera bruxellensis*: *Komagataeibacter intermedius* ratio and sugar concentration

Ratio of Yeast: Bacteria	% Sugar	Total Soluble solid (°Brix)			Alcohol (%v/v)			Acetic acid (%w/v)			Thickness of biofilm (mm)		
		Day2	Day4	Day6	Day2	Day4	Day6	Day2	Day4	Day6	Day2	Day4	Day6
1:1	10	10.01^{D}	8.50^{D}	5.73^{D}	0.30^{C}	0.50^{H}	2.31^{D}	0.12^{F}	1.20^{E}	2.46^{F}	1.24^{C}	2.00^{C}	6.00^{A}
	12	12.15^{C}	9.00^{D}	6.03^{C}	0.70^{C}	1.81^{F}	2.25^{D}	0.18^{F}	1.20^{E}	2.75^{EF}	1.30^{C}	4.67^{A}	5.00^{A}
	14	13.80^{C}	10.14^{C}	7.32^{B}	0.51^{C}	2.46^{D}	3.67^{C}	0.18^{F}	1.20^{E}	2.99^{E}	1.35^{C}	3.50^{AB}	5.50^{A}
	16	15.80^{B}	12.15^{C}	7.52^{B}	0.51^{C}	2.05^{F}	4.12^{C}	0.18^{F}	1.80^{DE}	3.02^{E}	1.27^{C}	4.00^{A}	5.83^{A}
	18	17.20^{B}	13.19^{B}	8.50^{B}	0.53^{C}	2.21^{E}	4.83^{B}	0.18^{F}	2.42^{D}	3.87^{C}	1.30^{C}	3.38^{B}	5.94^{A}
	20	19.60^{A}	15.00^{B}	10.12^{A}	0.51^{C}	2.20^{E}	4.12^{C}	0.18^{F}	2.40^{D}	3.76^{C}	1.50^{C}	3.26^{B}	5.62^{A}
1: 2	10	9.10^{D}	7.71^{E}	6.35^{C}	1.12^{B}	1.30^{G}	2.55^{D}	2.40^{C}	2.76^{CD}	2.82^{D}	2.90^{B}	4.33^{A}	6.00^{A}
	12	10.25^{D}	9.07^{D}	6.10^{C}	1.40^{B}	1.43^{FG}	2.60^{D}	2.90^{B}	3.17^{C}	3.00^{D}	3.75^{A}	4.28^{A}	5.00^{A}
	14	13.50^{C}	11.73^{C}	6.10^{C}	1.10^{B}	1.57^{FG}	2.61^{D}	3.18^{B}	2.97^{C}	2.97^{D}	4.44^{A}	4.76^{A}	5.00^{A}
	16	15.75^{B}	13.00^{B}	7.50^{B}	1.08^{B}	1.91^{F}	2.75^{D}	3.26^{B}	4.01^{B}	4.07^{C}	4.13^{A}	4.15^{A}	4.15^{B}
	18	18.25^{A}	17.20^{A}	8.50^{B}	1.17^{B}	1.16^{G}	2.25^{D}	4.36^{A}	4.61^{A}	4.89^{B}	1.90^{BC}	2.94^{B}	3.83^{B}
	20	20.50^{A}	19.31^{A}	10.50^{A}	1.05^{B}	1.86^{F}	2.20^{D}	4.10^{A}	4.88^{A}	4.88^{B}	1.71^{C}	2.75^{B}	3.75^{B}
2:1	10	8.33^{F}	6.25^{E}	4.20^{D}	0.93^{B}	3.90^{C}	4.10^{C}	0.40^{E}	2.55^{D}	3.20^{D}	1.15^{C}	4.20^{A}	5.80^{A}
	12	9.13^{F}	6.50^{E}	5.45^{D}	1.00^{B}	3.85^{C}	4.16^{C}	0.53^{E}	2.73^{CD}	3.00^{D}	1.28^{C}	3.40^{AB}	5.70^{A}
	14	11.67^{C}	8.10^{D}	6.95^{C}	1.33^{B}	4.15^{C}	4.22^{C}	1.03^{D}	2.97^{C}	3.05^{D}	1.43^{C}	3.55^{AB}	5.50^{A}
	16	13.13^{C}	9.50^{D}	6.05^{C}	1.33^{B}	4.60^{B}	4.15^{C}	1.37^{D}	2.80^{C}	3.25^{D}	1.40^{C}	3.95^{AB}	5.50^{A}
	18	15.67^{B}	11.50^{C}	6.50^{C}	2.00^{A}	5.75^{A}	6.75^{A}	0.43^{E}	3.35^{C}	5.62^{A}	1.03^{CD}	1.00^{CD}	1.15^{C}
	20	15.33^{B}	11.50^{C}	10.50^{A}	2.33^{A}	5.75^{A}	7.00^{A}	0.50^{E}	3.35^{C}	5.60^{A}	0.45^{D}	0.56^{D}	0.73^{D}

Values with different superscript A,B,C,.. in a column represent significant difference ($p < 0.05$)

From Table 1, an increase in acetic acid and alcohol contents in Kombucha were higher with a shorter fermentation time than the previous research of Gaggia *et al*. (2019), which reported that acetic acid and alcohol contents in Kombucha from black tea were 0.165 and 0.064% at day 7 of fermentation and increased to 0.489 and 0.114 at day 14 of fermentation. The results from this study were agreed with the report of Kumar and Joshi (2016) which explained that the production of acetic acid in black tea solution adjusted to contain 5-15% sugar concentration occurred in aerobic fermentation at 20-30 °C during 6-10 days. According to Petrovic et al. (1995-1996), the suitable fermentation time was seven days since the product had not too sour taste.

Kombucha from black tea fermentation of this study using different yeast and bacterial strains from other studies. The results showed that there was a good response of *Dekkera bruxellensis* and *Komagataeibacter intermedius* to sugar resulted in proper growth and biofilm production since day 2 of fermentation which usually appeared at day 3 (Kaewkod *et al*., 2019) and day 4 of fermentation (Valentin,

1930a,b; Sievers *et al*.,1995). In addition, when extended the fermentation time, the thickness of biofilm was increased, which was good to protect microbial contamination moving into the bottom of the fermentation medium (Greenwalt *et al*., 2000).

Figure 4 Appearance of biofilm at day 6 of fermentation using 10% sugar concentration and the ratio of yeast: bacteria as 1:1, 1:2, and 2:1 (from left to right)

From Figure 1, it was seen that using the amount of bacterial ratio equal to (1:1) or higher than (1:2) that of yeast affected the appearance of biofilm, which was creamy smooth. While increasing the amount of yeast ratio higher than bacteria (2:1), the appearance of biofilm was rough and bumpy spreading around the piece of biofilm. This appearance was associated with the more carbon dioxide production when compared to using other yeast: bacterial ratios as the results shown in Table 1.

5. Conclusion

Study of Kombucha production from black tea using *Dekkera bruxellensis* and *Komagataeibacter intermedius* as a symbiotic culture of yeast and bacteria, which was different from other researchers, showed that the results of fermentation at 10% sugar concentration with the ratio of yeast: bacteria as 1:1 agreed with those of fermentation with pure culture (Mankasetkit & Bowornsombat. 2017.; Zhiwei *et al*., 2010). However, different adjustments of the ratio of yeast: bacteria directly resulted in different qualities and fermentation times of the product. The information from this study will be beneficial to the commercial production of Kombucha for the entrepreneur to apply for improving fermentation efficiency.

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