Variation of the Content and Antioxidant Activity of Active Substances in the Kombucha Fermentation of Mulberry Leaves

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Abstract. With the increasing emphasis on healthy living, Kombucha beverages are becoming more and more popular. Recently, fermented Kombucha with different materials and investigated the efficacy have become hot topics. In this paper, mulberry leaves were used as substrates for Kombucha fermentation. The active substances contents and antioxidant activities of the fermented mulberry leaf infusion were significantly enhanced. After being fermented for 10 days, the total phenolic substances and total flavonoids in the Kombucha fermented mulberry leaf infusion were 7.38 and 12.24 times higher than those in the unfermented mulberry leaf infusion, respectively. While the mulberry leaf infusion that fermented for 6 days had the highest antioxidant capacity. The Ferric (Fe^{3+}) reducing power, $ABTS^{+}$ radical scavenging capacity, and hydroxyl radical (·OH) scavenging capacity were enhanced by 90.3%, 21.82%, and 46.2%, respectively. In conclusion, after being fermented by Kombucha, the antioxidant properties of mulberry leaf infusion were significantly improved, which will contribute to expanding the subsequent development and application of mulberry leaf.

Keywords: Mulberry leaf, Kombucha, Antioxidant activity, Total phenols, Total flavonoids.

1. Introduction

Mulberry leaf (ML), a kind of medicinal and edible plant, is rich in isoquercitrin, quercetin, choline, amino acids, sugars, flavonoids, alkaloids, organic acids, vitamins, phenolic substances, and other active ingredients.

Kombucha is a fermented beverage made by symbiotic fermentation of bacteria and yeast, which is popular worldwide and has been consumed in China, South Korea, Japan, the Philippines, and Russia for many years. Under the interaction of acetic acid bacteria and yeast, Kombucha produces a large number of active ingredients such as organic acids, polyphenols, vitamins, and minerals. Therefore, Kombucha has significant antioxidant, anti-cancer, antibacterial, anti-inflammatory, anti-diabetes, and other effects.

Recently, much research has been conducted to improve the flavor and function of Kombucha. Muhialdin et al found that Kombucha fermentation could obtain optimal antioxidant activity and phenolic content in a very short period and some of the biochemical properties of the Kombucha would reach the highest level within seven days of fermentation [1-2]. The soy whey broth fermented by Kombucha had the highest bacteriostatic efficiency against *Staphylococcus aureus* [3]. The concentration of total flavonoids and capacity of oxygen radical scavenging of the yellow slurry water, waste liquid from soybean product processing, were enhanced obviously after being fermented by Kombucha [4].

Given the increasing popularity and demand for Kombucha beverages, extensive scientific research on Kombucha is becoming a hot topic. Many different kinds of plants, like various fruits, crops, or herbal extracts, have been used for Kombucha fermentation to meet the diverse needs of consumers [5]. The diversity of raw materials provides Kombucha with diverse flavors and biological activities. This study using mulberry leaves as raw materials of Kombucha conducted detailed research on the changes of polyphenols and flavonoid contents in the fermentation broth, as well as the changes in antioxidant activity, including total reducing power, hydroxyl radical scavenging ability and ABTS radical scavenging ability. This paper will provide references to the recycling and development of mulberry leaves as functional foods.

2. Materials and methods

2.1. Materials

Mulberry leaves were purchased from the local market. After being water washed and drained the surficial water, the mulberry leaves were dried at 60℃ to constant weight and then pulverized to 20-40 mesh. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), rutin, gallic acid (≥98%), Folin-Ciocalteu regent, $K_3Fe(CN)_6$, salicylic acid were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). $FeSO_4$, $Al(NO_3)_3$, TCA, and $FeCl_3$ were obtained from Sinopharm Chemical Reagent Co., Ltd (Beijing, China).

2.2. Preparation of Kombucha inoculum

The Kombucha starter was kept in our laboratory. The preparation of Kombucha inoculum was conducted as follows: 5g of black tea leaves, was soaked in 500 ml hot water (≥90℃) for 20 min, and then taken the filter. Then, 10% sugar (v/v) was added to the infusion. After cooling, 10 % (v/v) Kombucha starter was inoculated aseptically and cultivated at 28-30℃ for 3 days.

2.3. Kombucha fermentation using Mulberry Leaf infusion

Taken 5g of crushed and dried mulberry leaves, added 10 times the volume of distilled water and 10% sugar, and mixed well. Then sterilized for 20 min at 115℃ and cooled for later use. Take 10% volume of Kombucha broth and 1 piece of bacterial membrane from the prepared Kombucha inoculum and inoculate them into the cooled mulberry leaf infusion medium. Then statically cultivated at 28-30℃ for 12 days.

2.4. Analysis of the Kombucha fermentation process

The pH and reduced sugar content of the fermentation broth were determined every 2 d. The pH was determined by audiometers. The 3,5-Dinitrosalicylic acid (DNS) method was used for the determination of reducing sugar content in the Kombucha fermented mulberry leaf infusion taking glucose as a standard [6].

2.5. Assays of active substances in Kombucha

The determination of total polyphenols and total flavonoids in the Kombucha fermentation broth of mulberry leaves was determined by a UV-Vis Spectrophotometer (754PC, Jinghua Science and Technology Co., Ltd., Shanghai, China).

Total phenolic substances in the Kombucha were determined by the Folin-Ciocalten method taking gallic acid as standard. The absorbance (A) of different concentrations of gallic acid was measured at 760 nm. The standard curve was plotted with the absorbance ($A_{760\ nm}$) as the vertical coordinate and the concentration of gallic acid (C) as the horizontal coordinate. The total phenolic substance content in test samples then be calculated.

The content of total flavonoids was determined by the $Al(NO_3)_3$ method using rutin as the standard. The absorbance (A) of different concentrations of rutin was measured at 510 nm. The standard curve was plotted with the absorbance ($A_{510\ nm}$) as the vertical coordinate and the concentration of rutin (C) as the horizontal coordinate. The total flavonoid content in the sample was calculated from the standard curve.

2.6. Analysis of antioxidant activity

Ferric (Fe^{3+}) reducing power assay: The determination of reducing the power of Kombucha was conducted based on the description of Raaman with slight modification [7]. Added 1 mL of 0.2 mol/L (pH 6.6) phosphate buffer solution and 1 mL of 1% $K_3Fe(CN)_6$ to 1 ml of a sample of Kombucha fermented mulberry leaf infusion. The mixture was then reacted at 50℃ for 20 min. Then 1 mL of 10% TCA solution was added and centrifuged at 6000 r/min for 10 min. Added 3 mL of deionized water and 0.5 mL of 0.1% $FeCl_3$ solution into 1.0 mL of the supernatant sequentially, then mixed

evenly and put at room temperature for 10 min. Measured the absorbance of the solution at 700 nm by a UV-Vis Spectrophotometer (754PC, Jinghua Science and Technology Co., Ltd., Shanghai, China). Distilled water was used as a blank control.

Hydroxyl radical (·OH) scavenging capacity [8]: Mixed 2 mL samples with 2 mL of 6 mmoL/L $FeSO_4$ solution and H_2O_2 solution well and put at room temperature for 10 min. Added 2 mL of 6 mmol/L salicylic acid solution and incubated in a water bath of 37℃ for 1 h. The absorbance of A_m at the wavelength of 510 nm was measured. A_0 was determined using deionized water as the reference solution, and the absorbance value of A_n can be determined using the same volume of distilled water to replace the salicylic acid solution. The hydroxyl radical scavenging rate was calculated according to the following formula.

$$\text{hydroxyl radical scavenging rate}/\% = [A0-(Am-An)]/A0\times 100\% \quad (1)$$

Where, A_0 and A_m are the absorbance value of distilled water and sample, respectively. A_n is the absorbance value measured with distilled water replacing salicylic acid.

$ABTS^+$ radical scavenging capacity: According to the general method make slight modifications. Mixed 7mmol/L ABTS and 2.45 mmol/L $K_2S_2O_8$ in a 1:1 ratio (v/v) and let stand at room temperature and dark for 12-16 h. Diluted the $ABTS^+$ radical solution with phosphate buffer (pH 7.4) to obtain the $ABTS^+$ test solutions which had an absorbance of 0.7±0.02 at 734 nm. Added 10μl sample to 190μl $ABTS^+$ test solution, mixed evenly, and let stand for 6 min at 30℃. Then measured the absorbance at 734 nm. Following the above method, 10μl of phosphate buffer (pH 7.4) was added with 190μl of $ABTS^+$ test solution and mixed well. The absorbance was recorded as A_1. The clearance rate can be calculated through the following formula.

$$ABTS^+ \text{ radical scavenging rate}/\% = (A_1-A)/A_1\times 100\% \quad (2)$$

Where, A, A_0 and A_1 are the absorbance of the $ABTS^+$ test solution, blank group and sample, respectively.

2.7. Statistical analysis

All experiments were conducted in triplicates and the data were presented as the mean ± standard deviation. Software Origin 8.5 was used to analyze the results.

3. Results and discussion

3.1. The fermentation process of Kombucha with mulberry leaf

During the fermentation, it can be seen that the pH value of the Kombucha decreased rapidly. After being fermented for 6 d the pH value of the fermentation broth decreased to 3.46±0.19 from the initial value of 5.43±0.24. It was mainly because as the microorganisms in the Kombucha grew a variety of organic acids were generated[9]. The generation of organic acid led to a rapid decrease in the pH value of the fermentation broth. However, after the 6th day of fermentation, the reduction rate of pH slowed down and decreased to 2.88±0.09 at the 12th day of the fermentation. For reducing sugar content, the initial concentration was 113.27±8.66 g/L. It decreased slowly at the beginning of fermentation, leading to the following two possible reasons. On the one hand, the growth of yeasts in the Kombucha would consume a part of reducing sugar. On the other hand, the enzymes secreted by *Saccharomyces cerevisiae* and *Lactobacillus* could decompose the cell wall of mulberry leaves, thus releasing a part of reducing sugar. These two aspects reasons would alleviate the decreasing speed of reducing sugar in the fermentation broth. However, from the 2nd day of fermentation, the reduced sugar concentration decreased rapidly, and decreased slowed down after the 8th day of fermentation (Figure 1).

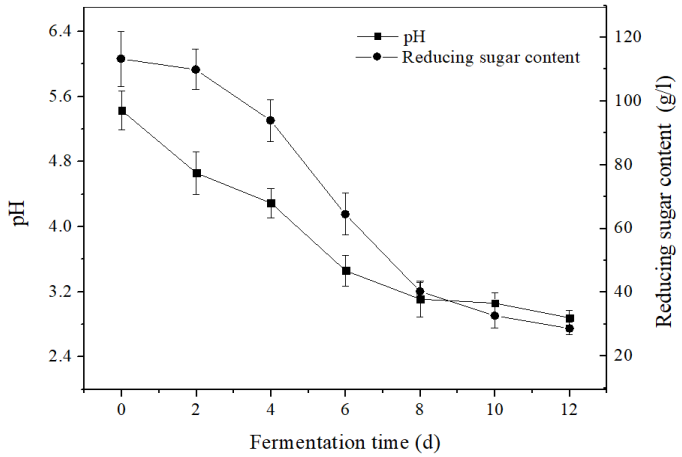

Fig. 1 Changes of pH and reducing sugar content during Kombucha fermentation of mulberry leaves.

3.2. Variation of phenolic substances and flavonoids content during Kombucha fermentation

The initial concentrations of total phenolic substances and total flavonoids in the fermentation infusion of mulberry leaves before fermentation were only 180.6±20.16 mg/L and 79.15±21.33 mg/L, respectively. Their concentrations steadily grew with time and peaked on the 10th day, which was 1332.71±70.24 mg/L and 968.42±68.49 mg/L, respectively. It can be seen that the contents of total phenolic substances and total flavonoids in the Kombucha were 7.38 and 12.24 times higher than those in the unfermented mulberry leaves infusion, respectively. The enhanced contents were owing to the microbial transformation and depolymerization of compounds. It has been reported that in many plants there were many covalent binding between phenolics and the structural components of the cell wall, including cellulose, hemicellulose, and lignin. However, these bonds can be hydrolyzed by hydrolase secreted by microorganisms and release soluble free phenols that have higher bioactivity. For example, Bei et al. pointed out that the amylase, pectinase, xylanase, cellulase, protease, and lipase secreted by *Monascus anka* could weaken the ether bonds between the components on the cell wall and the conjugated or bound phenolic substances, thereby facilitating the extraction of phenolic substances (Figure 2) [10].

In this study, the yeasts in Kombucha grown rapidly at the early stage of fermentation, which would promote the destruction of mulberry leaf cell wall structure and the degradation of some macromolecular active substances, resulting in the production of small molecule phenolic and flavonoid substances [11-12]. Xiao et al. also reported that *Lactobacillus plantarum* could significantly increase the total flavonoid content and antioxidant capacity of soy whey [13].

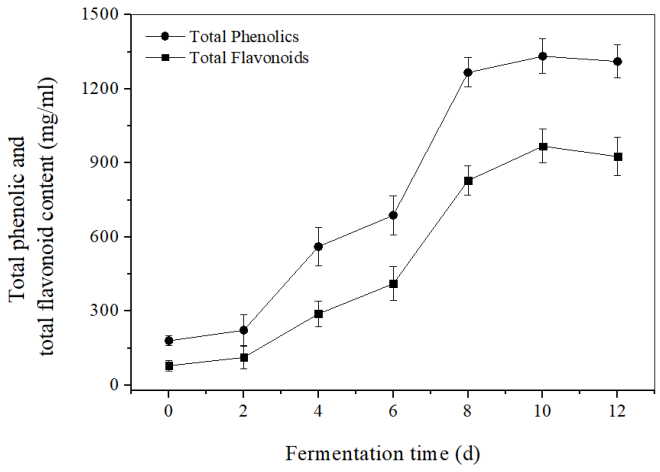

Fig. 2 Changes of total phenolic substances and total flavonoids in the fermentation products of Mulberry leaf Kombucha

3.3. In vitro antioxidant activity of Kombucha fermentation broth of mulberry leaf

Antioxidants would lose their activity by providing electrons to transform free radicals into stable molecules. In this paper, three antioxidant approaches were applied to estimate the antioxidant activity variation of mulberry leaf infusion after Kombucha fermentation.

Ferric (Fe^{3+}) reducing power denotes the total antioxidant capacity, which is an important indicator of the ability of antioxidant substances to provide electrons. Many studies have confirmed that antioxidant activity and reducing power are closely related to each other. In this study, the antioxidant properties of all the antioxidant substances in the Kombucha fermented mulberry leaf infusion were characterized in terms of Ferric (Fe^{3+}) reducing power. As shown in Fig. 3a, the Ferric (Fe^{3+}) reducing power was increased with the prolongation of the fermentation time and reached a maximum value of 0.539±0.037 after fermentation for 6 d. The hydroxyl radical scavenging capacity of the Kombucha fermented mulberry leaf infusion was investigated which indicated that the·OH scavenging capacity showed a tendency to first increase and then decrease with the fermentation time (Fig. 3b). The maximum value (67.81±2.87%) was obtained on the 6th day of fermentation, which was 46.2% higher than that at the beginning of fermentation. However, as the fermentation time continued to extend, the hydroxyl radical scavenging rate decreased slightly. As for the ABTS radical scavenging capacity, the highest value of 44.16±3.04% was achieved also on the 6th day of fermentation, which was increased by 21.82% compared to that before the fermentation. In conclusion, the antioxidant activity of mulberry leaf infusion during the Kombucha fermentation process showed similar trends when evaluated by the three methods. Considering the significant increase in both total phenolic substances and total flavonoid contents during Kombucha fermentation, it could be deduced that they had a positive correlation with the increase in antioxidant activity of fermented mulberry leaf infusion. However, continued extension of fermentation time would cause a decrease in the antioxidant activity of the mulberry leaf infusion. It might be attributed to two reasons. On the one hand, along with the growth of yeast, Kombucha fermentation entered a stable and declining phase. On the other hand, due to the presence of oxygen, the antioxidant substances were decomposed or undergo other chemical reactions, resulting in a decrease in antioxidant activity [14].

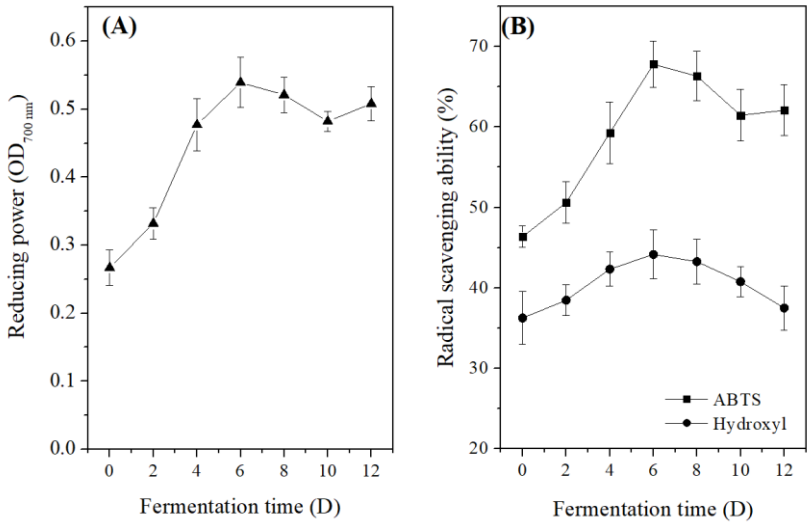

Fig. 3 Antioxidant properties of the fermentation broth of Mulberry leaf Kombucha
(A) Ferric (Fe^{3+}) reducing power; (B) Radical scavenging ability (%).

4. Conclusion

Mulberry leaves are typically edible and medicinal plants, rich in nutrition and high in medicinal value. In this study, mulberry leaves were taken as a new substrate for Kombucha to ferment. During the fermentation, the pH value and total reducing sugar content were decreased obviously from the

beginning of fermentation, on the contrary, the content of total phenolic substances and total flavonoid were increased significantly with the fermentation time. After 10 days of fermentation, the contents of them were enhanced by 6.38 and 11.24 times than those before fermentation, respectively. The antioxidant activity of the mulberry leaf Kombucha fermentation broth was increased first and then decreased with the fermentation time. Generally speaking, the 6-day fermentation broth of mulberry leaf had the highest antioxidant activity. In sum, Kombucha fermentation of mulberry leaves would not only enhance the content of active substances but also improve the antioxidant capacity. The work of this paper would provide a new way for the high-value utilization of mulberry leaves and also new ideas for developing novel flavors and functional Kombucha beverages.

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