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Influence of starter cultures on the antioxidant activity of kombucha beverage

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ABSTRACT

This paper investigates the influence of starter cultures, obtained from kombucha isolates, on the antioxidant activity of kombucha beverages. Three starter cultures were used as follows: (1) mixed culture of acetic bacteria and *Zygosaccharomyces* sp. (SC1); (2) mixed culture of acetic bacteria and *Saccharomyces cerevisiae* (SC2); as well as (3) native local kombucha. The starter cultures were added to black and green tea sweetened with 7% of sucrose. Fermentation was carried out at 28 °C for 10 days. Antioxidant activity to hydroxyl and DPPH radicals was monitored. Kombucha beverage on black tea has shown the highest antioxidant activity to both types of radicals with starter SC1, while the green tea beverage has shown the highest activity with native kombucha. The main reason for the different antioxidant activities, beside tea composition, was ascribed to differing production of both vitamin C and total organic acids in the investigated systems.

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1. Introduction

Kombucha is a symbiosis of several yeast strains and acetic acid bacteria. It is known under a number of trivial names, such as red tea fungus, champignon de longue vie, ling zhi, kocha kinoko, chainii grib, chainii kvass and many others (Hartman, Burleson, Holmes, & Geist, 2000). The dominant bacterium is *Acetobacter xylinum*, while the yeasts belong to the genera *Zygosaccharomyces, Schizosaccharomyces, Saccharomyces, Saccharomycodes, Candida, Pichia, Brettanomyces* and *Torulopsis* (Dufresne & Farnworth, 2000; Teoh, Heard, & Cox, 2004). The microbiological composition depends on the culture origin. During fermentation, *A. xylinum* produces a thin cellulose film where a cell mass of bacteria and yeasts is attached. It is a fungus-like mixture of cellulose and microorganisms (Sreeramulu, Zhu, & Knol, 2000).

Under aerobic conditions, kombucha symbiosis is capable of converting a very simple substrate (sucrose and black or green tea), over a period of 7–10 days, into a slightly carbonated, mildly sour and refreshing beverage. This beverage is composed of sugars, gluconic, glucuronic, L-lactic, acetic, malic, tartaric, malonic, citric, and oxalic acid, as well as ethanol, 14 amino acids, water soluble vitamins, antibiotically active matters and some hydrolytic enzymes (Balentine, Wiseman, & Bouwens, 1997; Bauer-Petrovska & Petrushevska-Tozi, 2000; Chen & Liu, 2000; Danielova, 1957; Hesseltine, 1965; Kappel & Anken, 1993; Pasha & Reddy, 2005; Steiger & Steinegger, 1957).

It has been reported that the kombucha beverage helps digestion, gives relief from arthritis, acts as a laxative, prevents microbial infections, helps in combating stress and cancer and vitalizes the physical body, etc. It is believed that this beverage enhances immunity (Dufresne & Farnworth, 2000). Prevention of microbial infections has been demonstrated against broad spectra of microorganisms, such as *Escherichia coli, Helicobacter pylori, Staphylococcus aureus, Salmonela cholerasius* serotype *typhymurium and Bacillus cereus* (Greenwalt, Steinkraus, & Ledford, 2000; Sreeramulu et al., 2000).

Activity of kombucha on the traditional carbon source sucrose was investigated by several authors (Dufresne & Farnworth, 2000; Reiss, 1994; Sievers, Lanini, Weber, Schuler-Schmid, & Teuber, 1995; Teoh et al., 2004) and main pathways of conversion of sucrose into numerous products were determined. In addition to sucrose, the application of any other sugar (lactose, glucose or fructose) is possible.

Many beneficial effects to the human body can be achieved using substances with antioxidative properties. Substrates for kombucha fermentation contain antioxidants which originated from tea leaves. These are mainly polyphenols, especially catechins, which belong to the flavanols group (Graham, 1992; Mukhtar & Ahmad, 2000). Beside polyphenols, kombucha beverage contain metabolites, like vitamin C, B_2, B_6, and catalase, which have a free-radicals trapping ability (Djilas, Čanadanović-Brunet, & Ćetković, 2002) or can act synergistically with antioxidants like citric acid (Rižner Hraš, Hadolin, Knez, & Bauman, 2000).

The study of Jayabalan, Subathradevi, Marimuthu, Satishkumar, and Swaminathan (2008) demonstrated that kombucha tea, prepared from green tea, black tea and tea waste material developed excellent antioxidant activities. The authors pointed out a great potential usage of waste tea material for the preparation of the kombucha beverage with a high antioxidant capacity. However,

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many practical aspects, related to the working conditions, must be solved before the production of this kombucha beverage, at a large scale, could succeed. Having an optimal and stable (controllable) starter culture is one of the most important conditions. Recent investigations suggested the scaling up of kombucha beverage production using native kombucha as a starter culture (Malbaša et al., 2006). It can be assumed that kombucha fermentation on a larger scale could be easier to control when using starter cultures which contain a smaller number of microorganism species.

This article considers the influence of kombucha starter cultures on the antioxidant activity of kombucha beverages to certain free radicals. The starter cultures were created using kombucha isolates, while native kombucha was used for comparison.

2. Material and methods

2.1. Microorganisms

The local kombucha culture contains at least five yeast strains (*Saccharomycodes ludwigii, Saccharomyces cerevisiae, Saccharomyces bisporus, Torulopsis* sp. and *Zygosaccharomyces* sp.), which were determined in previous investigations (Markov, Malbaša, Hauk, & Cvetković, 2001). Primary kombucha bacterium belongs to the strains of the genus *Acetobacter* (Reiss, 1994; Sievers et al., 1995; Teoh et al., 2004).

Three different inoculums are used as starter cultures. These are:

– Fermentation liquid of native local kombucha culture (Control) after 7 days of fermentation.

– Starter culture (SC1) composed of local kombucha isolates: *S. cerevisiae* (approx. 5.8×10^4 cells/ml of substrate) and mixed culture of acetic acid bacteria (approx. 10^5 cells/ml of substrate).
– Starter culture (SC2) composed of local kombucha isolates: *Zygosaccharomyces* sp. (approx. 5.8×10^4 cells/ml of substrate) and mixed culture of acetic acid bacteria (approx. 10^5 cells/ml of substrate).

The number of yeasts and bacteria in SC1 and SC2 correlates to the number of microorganisms in the control.

The yeast isolates *Zygosaccharomyces* sp. and *S. cerevisiae* were chosen from five yeast isolates because they showed the highest and the lowest production of acids, respectively, in preliminary investigations. Thus the extremes were chosen.

2.2. Substrates

The kombucha culture (Control), SC1 and SC2 were all cultivated on two different substrates.

The substrate from black tea was prepared from 1 l of boiled tap water with 70 g of sucrose (forming 7% solution of sucrose) and 1.5 g of black tea (Indian tea, "Vitamin", Horgoš, Serbia). The tea was heated for 5 min at a temperature of 100 °C, then tea leaves were removed by filtration and the obtained solution was cooled to room temperature.

The substrate from green tea (Grüner Tee, Milford, Austria) was prepared under the same conditions as the substrate from black tea.

2.3. Fermentation

Fermentation was carried out at 28 °C for 10 days. Samples were taken periodically for further analysis. Fermentation was repeated three times.

2.4. Methods of analysis

2.4.1. Hydroxyl and DPPH radical antioxidant activity

Generation of hydroxyl radicals was conducted by the mixing of 0.2 ml 0.3 M DMPO (5,5-dimethyl-1-pyrroline-N-oxide), 0.2 ml 10 mM H_2O_2, 0.2 ml 10 mM Fe^{2+} and 0.2 ml of black or green tea. The reaction mixture was then transferred to a quartz ESR flat cell ER-160-FC for ESR analyses. ESR spectra were recorded after 5 min on a Bruker ESR-300 E spectrometer (Rheinstetten, Karlsruhe, Germany). To establish the influence of kombucha beverages on the formation and stabilization of hydroxyl radicals, 0.2 ml of fermentation samples were added to the appropriate mixture of reagents instead of black or green tea. The antioxidant activity AA_{OH} was calculated as the percentage of reduction of hydroxyl radical concentration.

Solutions with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were prepared by mixing of 0.1 ml methanol, 0.6 ml 0.4 M DPPH and 0.1 ml of black or green tea. To establish the influence of kombucha beverages on the stabilization of DPPH radicals, 0.1 ml of the fermentation samples were added to the appropriate DPPH solution instead of black or green tea. The antioxidant activity AA_{DPPH} was calculated as the percentage of reduction of DPPH radical concentration.

All chemicals for ESR analysis were purchased from Sigma Chemicals Co. and used without further purification.

All analyses were performed in triplicate.

2.5. Determination of pH, total acid, citric acid, vitamin B_2 and vitamin C content

pH values were measured by an electric pH-metre (Iskra, MA 5713, Kranj, Slovenia).

Total acid content was determined by titration with sodium hydroxide (0.09551 M) using phenolphthalein as indicator.

Citric acid content was quantified with Megazyme (Ireland) kit (Cat. No. K-CITR).

Vitamin B_2 was determined using the HPLC technique. A NOVA-PAK™ column (Waters, USA), having dimensions 100×8 mm, diameter 10 μm and with cartridge RP-Radial PAK™, was used. As a mobile phase, ammonium acetate (5 mM) and methanol 7.7:3 (v/v), at pH 3, were applied. All chemicals were HPLC purity grade. The flow rate was 2 ml/min and the loop volume was 20 μl. Vitamin B_2 was detected by a fluorescence detector; excitation and emission were recorded at both 450 and 530 nm. Shimadzu-Japan software was used. The samples for HPLC analyses were prepared by filtering through a hydrophilic membrane filter, which had a pore diameter of 22 μm.

Vitamin C content was quantified with Megazyme (Ireland) kit (Cat. No. K-ASCO).

All analyses were performed in triplicate.

3. Results and discussion

3.1. Development of fermentation

Kombucha fermentation was monitored over a period of 10 days by measuring pH (Fig. 1). The same pattern in changes of pH, in addition very similar pH values were noticed for the Control, SC1 and SC2 series of samples. Although the initial pH value of green tea was lower compared to black tea, the pH values in samples with green tea were higher during the fermentation process (Fig. 1b). This difference between the pH values of the substrates could be the consequence of differences in chemical composition of black and green tea (Mukhtar & Ahmad, 2000). The pH pattern during fermentation was as expected and it had the same shape

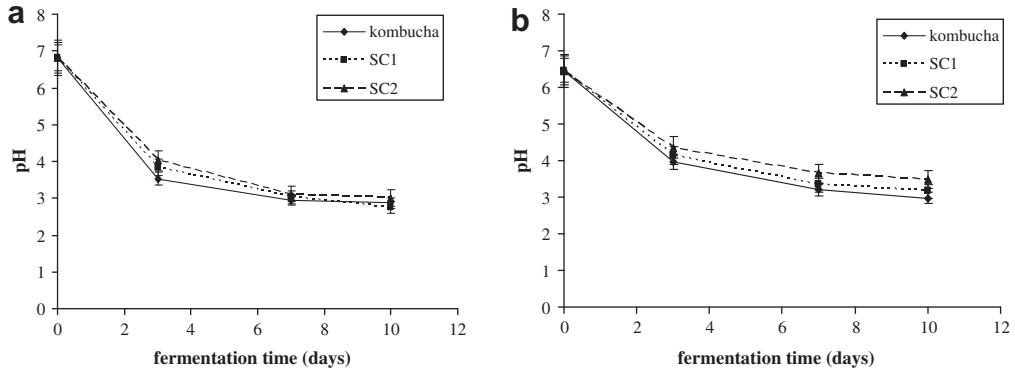

Fig. 1. pH changing during fermentation process on different substrates: (a) black tea; (b) green tea.

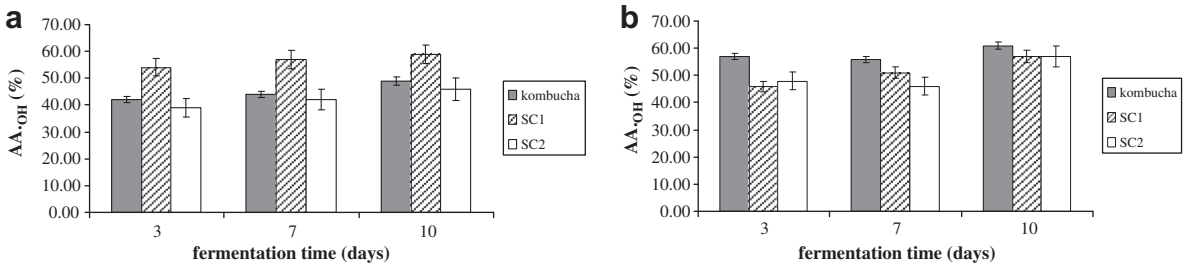

Fig. 2. Antioxidant activity to hydroxyl radical of different kombucha samples obtained on different substrates: (a) black tea; (b) green tea.

for both substrates. A very significant decrease of pH was noticed after three days and, thereafter, the changes of pH were very slow until the end of fermentation. This is the consequence of a buffering effect caused by synthesis of weak organic acids, which interact with the mineral matters from tea. These results are in accordance with a number of published data (Jayabalan et al., 2008; Lončar, Petrović, Malbaša, & Verac, 2000; Malbaša, 2004; Sreeramulu et al., 2000). It is important to say that products obtained using SC1 and SC2 were similar to the Control products. They were clear, slightly carbonated, with a typical odour and taste.

3.2. Antioxidant activity

The antioxidant activity of black and green tea is undisputable. The idea was to investigate the potentially higher antioxidant activity of fermented samples compared to the pure teas as samples blank. The samples obtained by fermentation with SC1 and SC2 were especially interesting, because it was proved that native kombucha culture produces the beverage with antioxidant properties (Jayabalan et al., 2008), even if the kombucha originates are from very different geographic regions. Hydroxyl and DPPH free radicals were chosen as the undesired species because they are two different types in terms of reactivity and origin. Hydroxyl radicals are very reactive, but DPPH radicals are relatively stable. Also, it is possible to generate hydroxyl radicals in the human body, while DPPH radicals are synthetic products, which are usually used for the investigation of antioxidant activity to relatively stable reactive species.

Antioxidant activity, $AA_{\cdot OH}$, of fermentative liquids and kombucha beverages on the hydroxyl radicals is shown in Fig. 2. A significant increase of the $AA_{\cdot OH}$ was observed in all cultivation mixtures after three days of fermentation (average value 47.7%) (Fig. 2). After that period, a very slight increase of the $AA_{\cdot OH}$ was observed, until the end of fermentation. It is obvious that the compounds produced by kombucha caused a significant effect. After comparison among the $AA_{\cdot OH}$ values for samples obtained on different substrates, but with the same starter cultures, it was possible to notice the differences, i.e. the average value of the $AA_{\cdot OH}$ is higher for the samples obtained from green tea inoculated with different cultures. Also, it was noticed that the highest average $AA_{\cdot OH}$ was obtained with the SC1 on the substrate with black tea (Fig. 2a), while the highest activity was achieved with the Control starter on the substrate with green tea (Fig. 2b). There could be several reasons for such antioxidant behaviour, but the main is again the fact that polyphenols composition and content in black and green tea are not the same (Graham, 1992; Mukhtar & Ahmad, 2000).

The dynamics of changes of AA_{DPPH} were almost completely different to the rate of changes of $AA_{\cdot OH}$. The only similarity between them was a significant increase after three days of fermentation (average value 48.7%), but continual decrease of AA_{DPPH} was found until the end of fermentation (Fig. 3).

Some similarities between antioxidant activities, $AA_{\cdot OH}$ and AA_{DPPH}, were noticed however. Both types of antioxidant activities achieved the highest values using SC1 on the substrate with black tea (Figs. 2a and 3a). The highest values were achieved with the Control on the substrate with green tea (Figs. 2b and 3b). The lowest antioxidant activities were obtained with SC2 on both substrates. On the substrate with green tea, using SC2 as an inoculum, AA_{DPPH} decreases significantly until the end of fermentation (Fig. 3b). This fact certainly does not recommend SC2 for kombucha beverage production.

The highest number of mixed cultures of acetic acid bacteria and yeasts was achieved with the Control at the end of the fermentation on both substrates. There were approx. 5.2×10^6 of bacterial cells/ml and 3.1×10^6 of yeast cells/ml of black tea substrate, and 4.9×10^6 of bacterial cells/ml and 2.8×10^6 of yeast cells/ml of green tea substrate. The microbial count at the end of fermentation of both SC1 and SC2 was slightly lower in comparison to the Control. The facts mentioned above indicate that the microbial count does not correlate to $AA_{\cdot OH}$ and AA_{DPPH}.

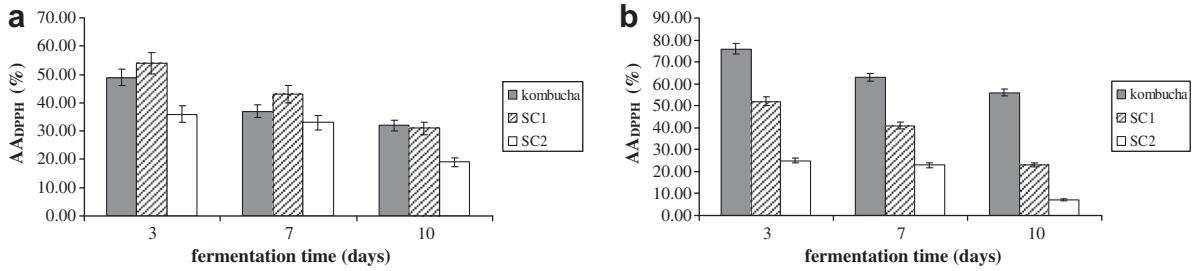

Fig. 3. Antioxidant activity to DPPH radical of different kombucha samples obtained on different substrates: (a) black tea; (b) green tea.

3.3. Vitamins C and B_2

Vitamin C is one of the very famous antioxidant vitamins. It helps to maintain healthy collagen in the skin, repairs damaged tissue, promotes healthy teeth and bones, and boosts the immune system. Vitamin C, as a free-radical fighter, helps ward off wrinkles and many illnesses linked to oxidation, including cataracts, arthritis, heart disease, and cancer (Du Toit, Volsteedt, & Apostolides, 2001). Vitamin C content showed a continual increase, which was monitored in systems with all starter cultures on both substrates (Fig. 4). It was possible to notice a correlation between the highest values of antioxidant activities and the quantity of vitamin C in some series of samples (Figs. 2–4). These are the samples of SC1 on black tea and the Control samples on green tea.

Vitamin B_2 is important because it is a strong antioxidant with immunoenhancing properties. It plays a central role in biological redox reactions. The measured quantities of vitamin B_2 (Fig. 5) agree with the contents of B_2 obtained in our previous investigations on similar systems (Malbaša, Maksimović, Lončar, & Branković, 2004). However, the antioxidant activities of the investigated systems do not correlate with the content of vitamin B_2. A comparison of the results shows that the highest values of vitamin B_2 were achieved in the Control samples on both kinds of tea (Fig. 5). Yeasts are responsible for the biosynthesis of B group vitamins. It is obvious that a symbiosis of several yeasts in the Control has greater capability to produce vitamin B_2 than SC1 and SC2 on their own (Malbaša et al., 2004).

3.4. Total acids and citric acid content

After analysis of the results of total acids content (Fig. 6) along with the results of antioxidant activity of the kombucha beverage to the hydroxyl and DPPH radicals (Figs. 2 and 3), an interesting correlation was noticed. For samples obtained from kombucha fermentation on black tea, there is a strong connection between the $AA_{\cdot OH}$ and AA_{DPPH}, on one hand, and total acids content, on the other. Samples obtained with SC1 possess the highest total acids content, higher than the control samples (Fig. 6a). Green tea samples, generally, have a higher content of total acids than samples obtained on black tea, while the Control series have the highest content (Fig. 6b). There is also a correlation between the $AA_{\cdot OH}$ and AA_{DPPH}, on one hand, and total acids content, on the other, in kombucha samples obtained on green tea, but this correlation does not follow the same pattern as for black tea samples.

Citric acid could have synergistic effects to some antioxidant mixtures like rosemary extract (Rižner Hraš et al., 2000). This was the reason for determination of citric acid in kombucha samples. The results show a very small amount of citric acid in the total acids (average 2.5%), which indicates that citric acid in kombucha

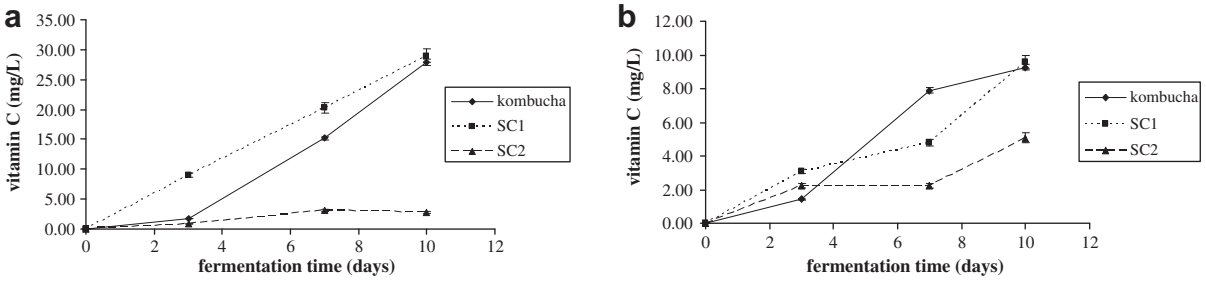

Fig. 4. Vitamin C content of different kombucha samples obtained on different substrates: (a) black tea; (b) green tea.

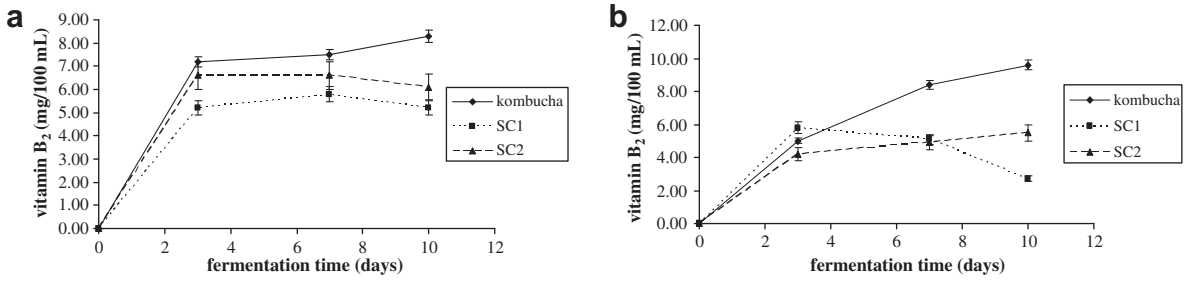

Fig. 5. Vitamin B_2 content of different kombucha samples obtained on different substrates: (a) black tea; (b) green tea.

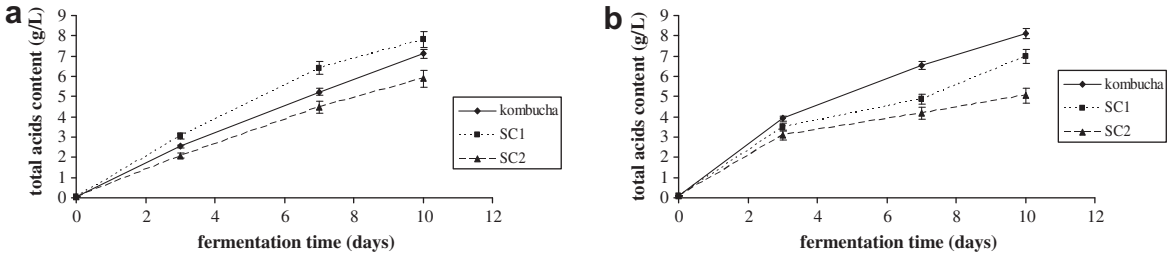

Fig. 6. Total acids content of different kombucha samples obtained on different substrates: (a) black tea; (b) green tea.

samples does not have a significant impact on their antioxidant activity.

4. Conclusion

These investigations proved that the fermented beverages can be successfully produced by application of starter cultures composed of isolates from native kombucha. During the fermentation, valuable compounds are generated, which gives kombucha beverages a strong antioxidant character. Also, the following particular conclusions can be drawn:

- the application of different kombucha starters causes a development of different antioxidant activities on both substrates;
- the highest $AA_{\cdot OH}$ and AA_{DPPH} on black tea was obtained using SC1, while on green tea it was achieved using the Control. The lowest $AA_{\cdot OH}$ and AA_{DPPH}, which was obtained with SC2, does not recommend this starter for further application;
- antioxidant activities correlate with the total acids and vitamin C content.

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References

Balentine, D. A., Wiseman, S. A., & Bouwens, L. C. (1997). The chemistry of tea flavonoids. *Critical Review in Food Science and Nutrition, 37*, 693–704.

Bauer-Petrovska, B., & Petrushevska-Tozi, L. (2000). Mineral and water soluble vitamins content in the kombucha drink. *International Journal of Food Science and Technology, 35*, 201–205.

Chen, C., & Liu, B. Y. (2000). Changes in major components of tea fungus metabolites during prolonged fermentation. *Journal of Applied Microbiology, 89*, 834–839.

Danielova, L. T. (1957). K khimicheskomu sostavu i fiziko-khemicheskim svoistvam kulturalnoi zhidkosti chainogo gryba. *Trudy Erevanskogo Zooveterinarnogo Instituta, 22*, 111–121.

Djilas, S. M., Čanadanović-Brunet, J., & Ćetković, G. S. (2002). Antioxidants in food. *Chemical Industry, 56*(3), 105–112.

Dufresne, C., & Farnworth, E. (2000). Tea, kombucha, and health: A review. *Food Research International, 33*(6), 409–421.

Du Toit, R., Volsteedt, Y., & Apostolides, Z. (2001). Comparison of the antioxidant content of fruits, vegetables and teas measured as vitamin C equivalents. *Toxicology, 166*(1–2), 63–69.

Graham, H. N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Preventive Medicine, 21*, 334–350.

Greenwalt, C. J., Steinkraus, K. H., & Ledford, R. A. (2000). Kombucha, the fermented tea: Microbiology, composition, and claimed health effects. *Journal of Food Protection, 63*(7), 976–981.

Hartman, A. M., Burleson, L. E., Holmes, A. K., & Geist, C. R. (2000). Effects of chronic kombucha ingestion on open-field behaviors, longevity, appetitive behaviors, and organs in C57-BL/6 mice. A pilot study. *Nutrition, 16*(9), 755–761.

Hesseltine, C. W. (1965). A millenium of fungi. Food and fermentation. *Mycologia, 57*(2), 148–167.

Jayabalan, R., Subathradevi, P., Marimuthu, S., Satishkumar, M., & Swaminathan, K. (2008). Changes in free-radical scavenging ability of kombucha tea during fermentation. *Food Chemistry, 109*, 227–234.

Kappel, T., & Anken, R. H. (1993). The tea mushroom. *The Mycologist, 7*, 12–13.

Lončar, E. S., Petrović, S. E., Malbaša, R. V., & Verac, R. M. (2000). Biosynthesis of glucuronic acid by means of tea fungus. *Nahrung, 44*(2), 138–139.

Malbaša, R. V. (2004). *Investigation of antioxidant activity of beverage from tea fungus fermentation*. Ph.D. thesis (in Serbian). University of Novi Sad, Faculty of Technology, Novi Sad.

Malbaša, R. V., Maksimović, M. Z., Lončar, E. S., & Branković, T. I. (2004). The influence of starter cultures on the content of vitamin B_2 in tea fungus beverages. *Central European Journal of Occupational and Environmental Medicine, 10*(1), 79–83.

Malbaša, R., Lončar, E., Djurić, M., Klašnja, M., Kolarov, Lj., & Markov, S. (2006). Scale-up of black tea batch fermentation by kombucha. *Food and Bioproducts Processing, 84*(C3), 193–199.

Markov, S. L., Malbaša, R. V., Hauk, M. J., & Cvetković, D. D. (2001). Investigation of tea fungus associations. I. The yeasts. *Acta Periodica Technologica, 32*, 133–138.

Mukhtar, H., & Ahmad, N. (2000). Tea polyphenols: Prevention of cancer and optimizing health. *The American Journal of Clinical Nutrition, 71*, 1698S–1702S.

Pasha, C., & Reddy, G. (2005). Nutritional and medicinal improvement of black tea by yeast fermentation. *Food Chemistry, 89*, 449–453.

Reiss, J. (1994). Influence of different sugars on the metabolism of the tea fungus. *Zeitschrift fur Lebensmittel Untersuchung und Forschung, 198*, 258–261.

Rižner Hraš, A., Hadolin, M., Knez, Ž., & Bauman, D. (2000). Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbil palmitate and citric acid in sunflower oil. *Food Chemistry, 71*, 229–233.

Sievers, M., Lanini, C., Weber, A., Schuler-Schmid, U., & Teuber, M. (1995). Microbiology and fermentation balance in kombucha beverage obtained from a tea fungus fermentation. *Systems Applied Microbiology, 18*, 590–594.

Sreeramulu, G., Zhu, Y., & Knol, W. (2000). Kombucha fermentation and its antimicrobial activity. *Journal of Agricultural and Food Chemistry, 48*(6), 2589–2594.

Steiger, K. E., & Steinegger, E. (1957). Uber den teepilz. *Pharmaceutica Acta Helvetica, 32*, 133–154.

Teoh, A. L., Heard, G., & Cox, J. (2004). Yeast ecology of kombucha fermentation. *International Journal of Food and Microbiology, 95*, 119–126.