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Symbiosis of acetic acid bacteria and yeast isolated from black tea fungus mimicking the kombucha environment in bacterial cellulose synthesis

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Abstract

The symbiotic effect of acetic acid bacteria and yeast on bacterial cellulose (BC) synthesis in kombucha was explored. Firstly, the optimal culture ratio of acetic acid bacteria and yeast was optimised through single factor and orthogonal test. The results showed that when *Komagataeibacter intermedius:Brettanomyces bruxellensis:Zygosaccharomyces bisporus* ratio was 1:10:10, and the inoculation amounts of *K. intermedius*, *B. bruxellensis*, and *Z. bisporus* were 10^4, 10^5, and 10^5 CFU/mL, respectively, the yield of BC was the highest, and the dry basis was 5.51 g/L. It was determined that the metabolites of *B. bruxellensis* and *Z. bisporus* could promote the synthesis of BC by *K. intermedius*. In addition, the composition of yeast filtrate was analysed by amino acid analyser, gas chromatography-mass spectrometry (GC-MS), and high performance liquid chromatograph (HPLC). Results showed that 16 amino acids were detected in yeast filtrate, and cysteine was only detected in yeast filtrate. The increase in isoleucine before and after fermentation was the highest, which was 11.64 times that of the control group. The increase in aspartic acid and glycine were second and third, accounting for 60.00 and 41.67%, respectively. The main volatile substances were alcohols, accounting for 84.89%, of which the relative content of ethanol was the highest at 77.35%. The relative contents of 3-methyl-1-butanol and phenylethanol were also high, accounting for 4.13 and 3.14%, respectively. Tartaric, citric, and succinic acids were detected in the yeast filtrate. The chemical species did not change before and after fermentation, but the content decreased. Vitamins B_2 and B_6 were detected in yeast filtrate, and the species and content did not change significantly before and after fermentation. A theoretical basis for kombucha fermentation and BC synthesis was provided.

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Introduction

Biocellulose (BC) is an extracellular polysaccharide secreted by microorganisms that is insoluble in water, and has a white or milky white appearance. It is an unbranched long-chain repeating unit composed of β-D-glucose and β-1, 4-glycosidic bonds, with a distinct cellulose structure. BC contains a large number of hydroxyl groups. The hydrogen bonds formed by the available hydroxyl groups on the adjacent dextran chains make the dextran chains form insoluble lamellae (Ross *et al.*, 1991), which presents a unique three-dimensional network structure. Therefore, it was widely used in various fields, such as bone tissue engineering scaffolds (Bose *et al.*, 2012). However, the production of BC by *Gluconacetobacter xylinus* was costly (the culture medium accounts for about 30% of the total cost (Jozala *et al.*, 2016)) and low yield (Tian *et al.*, 2018; Gayathri and Srinikethan, 2019). When compared with *G. xylinus*, kombucha can use cheap tea syrup to ferment and produce BC, and its yield is several times that of *G. xylinus*.

Kombucha was originated in Northeast China during the Qin Dynasty in 220 B.C. (Jayabalan *et al.*, 2014). Kombucha is a beverage produced by the fermentation of yeast and bacteria (called a symbiotic culture of bacteria and yeast, also known as SCOBY) (Emiljanowicz and Malinowska-Pańczyk, 2020). In addition to tea, fruit and vegetable juices, herbal or plant extracts, and food processing by-products can also be used as fermentation raw materials of kombucha to produce healthy and functional products (Shahbazi *et al.*, 2018; Rahmani *et al.*, 2019). The

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main microorganisms of kombucha are yeasts (*Candida*, *Brettanomyces*, *Saccharomyces*, *Schizosaccharomyces*, and *Zygosaccharomyces*) and acetic acid bacteria (*Acetobacter* and *Gluconacetobacter*), and a few also contain lactic acid bacteria (*Lactobacillus*) (Nguyen *et al.*, 2015; Coton *et al.*, 2017; Zou *et al.*, 2021). Moreover, due to the high conversion rate of sugar in kombucha, more sugar can be used to produce BC (Sharma and Bhardwaj, 2019). Yeasts use sucrose to produce glucose and fructose, and produces ethanol through ethanol fermentation. Acetic acid bacteria use glucose and ethanol to produce gluconic acid and acetic acid. Bacteria can also produce BC through metabolism. The glucan bacillus xylanase is mainly responsible for the synthesis of cellulose matrix, and the BC produced is retained on the culture liquid surface. Lactic acid bacteria metabolise to produce lactic acid, but with the increase in ethanol concentration, the growth of lactic acid bacteria is inhibited (Martínez Leal *et al.*, 2018).

Kombucha produces BC, which mainly includes four enzymatic steps: phosphorylation of glucose, isomerisation of glucose-6-phosphate to glucose-1-phosphate, synthesis of uridine diphosphate glucose, and cellulose synthase reaction (Rajwade *et al.*, 2015). Glucose is converted into glucose-6-phosphate, glucose-1-phosphate, and uridine diphosphate glucose under the action of glucokinase, phosphoglucose mutase, and uridine triphosphate glucose-1-phosphate uridine transferase. Under the action of cellulose synthase, the product is transformed into branchless β-1, 4-D-glucan that is BC (Krasteva *et al.*, 2017; Reiniati *et al.*, 2017). *Komagataeibacter*, the dominant bacterium in kombucha, could use hydrocarbons at the *n*-decane or mineral oil-kombucha suspension interface to produce elastic BC, which had a good development prospect (Subbiahdoss *et al.*, 2022). Avcioglu *et al.* (2021) used *K. saccharivorans* LN886705, *Brettanomyces bruxellensis* MH393498, and *B. anomalus* KY103303 as kombucha fermenting strains for fermentation. The BC produced was 4.06 times higher than that of the medium, and had excellent characteristics such as ultra-high purity, high crystallinity, and high thermal stability.

Due to the growth and metabolism of yeasts, acetic acid bacteria, and other bacteria, kombucha is rich in bioactive substances, including organic acids (acetic, gluconic, glucuronic, citric, L-lactic, malic, and tartaric acid, *etc*.), sugars (sucrose, glucose, and fructose), water-soluble vitamins (B_1, B_2, B_6, B_{12}, and C), amino acids, ethanol, and minerals, so it has good functional characteristics, such as bacteriostasis and oxidation resistance (Jakubczyk *et al.*, 2020). Zhou *et al*. (2022) used black and green tea dregs as fermentation substrates to prepare kombucha, and studied the antioxidant activity of kombucha. The results showed that tea residue fermentation could significantly improve the antioxidant activity and polyphenolic content of tea fungus. In addition, green tea dregs showed a stronger effect than red tea dregs. However, the symbiotic mechanism between acetic acid bacteria and yeasts is not clear. At present, one explanation accepted by most people is that yeast provides ethanol for the growth of acetic acid bacteria, but it may be far more than that. Stadie *et al*. (2013) isolated two strains of yeasts (*Zygotorulaspora florentina* and *Saccharomyces cerevisiae*) and two strains of lactic acid bacteria (*Lactobacillus horde* and *L. nagelii*) from kefir grains, and the interaction between them was studied. The results showed that isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, valine, arginine, and vitamin B_6 secreted by yeast could promote the growth of lactic acid bacteria. Zoumpourtikoudi *et al.* (2018) added the cell free supernatant of *S. cerevisiae* to *Lactococcus lactis* and *Lactiplantibacillus plantarum* culture medium, respectively, and found that the number of living cells of *L. lactis* and *L. plantarum*, and lactic acid synthesis increased. The most abundant amino acids accumulating in the yeast conditioned medium were glutamine, alanine, glutamate, serine, and glycine, and various amino acids were added into the pure culture medium of lactic acid bacteria based on the corresponding contents. The growth conditions of *L. plantarum* and *L. lactis* were consistent with those in the presence of *S. cerevisiae*, thus indicating that the growth-promoting factors were amino acids (Ponomarova *et al.*, 2017).

In the previous work, we isolated and identified acetic acid bacteria and yeasts from the black tea fungus sold in China, and obtained four dominant bacteria, namely *Komagataeibacterium intermedius*, *Brettanomyces bruxellensis*, *Zygosaccharomyces bisporus*, and *Metschnikowia fructicola*. Through experiment, we found that the yield of BC in pure culture of acetic acid bacteria was much less than that in co-culture with yeast. In order to clarify the mechanism of yeast promoting the synthesis of BC by acetic acid bacteria, the best co-culture ratio of acetic

acid bacteria and yeast was first determined, and then the action mode of yeast on acetic acid bacteria was clarified. Finally, the action mechanism was explored to provide a theoretical basis for improving the yield of BC and reference for studying the interaction between species in microbial communities.

Materials and methods

Materials

Komagataeibacter intermedius, Brettanomyces bruxellensis, Zygosaccharomyces bisporus, and *Metschnikowia fructicola* were all isolated from kombucha sold in China. Glucose and disodium hydrogen phosphate dodecahydrate were purchased from Tianjin Tianda Chemical Experimental Factory. Yeast extract powder and peptone were purchased from Beijing Boxing Biotechnology Co., Ltd. Citric acid was purchased from Tianjin Karatton Chemical Co., Ltd. Agar was purchased from Shanghai Lanji Technology Development Co., Ltd. Natamycin was purchased from Aladdin Reagent Co., Ltd. Malt juice agar medium was purchased from Beijing Sanyao Technology Development Company. Cellulase (15,000 U/g), sodium citrate, ethanol, ninhydrin, ethylene glycol monomethyl ether, sodium borohydride, anhydrous sodium acetate, and trichloroacetic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. Methanol and acetonitrile were purchased from an American ASC company. Cetyltrimethyl ammonium bromide was purchased from Tianjin Body Chemical Co., Ltd. Amino acid mixed standard solution was purchased from Heguang Pure Medicine Industry Co., Ltd. Organic acid standard was purchased from Dr. Ehrenstorfer Company in Germany. Vitamin standard was purchased from Shanghai Yuanye Biotechnology Co., Ltd. Dahongpao Tea was purchased from Wuyishan, Fujian province.

Activation, inoculation, and cultivation of strains

Activation, inoculation, and cultivation of *K. intermedius* were as follows: Historian Schramm (HS) medium was prepared by adding 2% glucose, 0.5% peptone, 0.5% yeast extract, 0.115% citric acid, and 0.68% disodium hydrogen phosphate dodecahydrate to deionised water, and the pH was adjusted to 6.0 with acetic acid. HS plate culture medium was prepared by adding 0.1% natamycin and 1.8% agar on the basis of HS culture medium, and natamycin was added after the sterilisation of culture medium, and before the plate was poured. The strains were transferred to the HS plate medium on SW-CJ-2D ultra clean workbench (Yongguangming, Beijing, China) to obtain a single colony, inoculated with two rings of strains in 50 mL of HS medium, and incubated at 30°C for 24 h. The acetic acid bacteria in seed solution were counted by the dilution spread plate method. They were inoculated based on the ratio of 10^4 CFU/mL after counting. After inoculation, it was incubated in DH-360 electric constant temperature incubator (Zhong Xing, Beijing, China) at 30°C for 7 d. In the experiment of the influence of yeast filtrate with different fermentation times on the yield of BC, 50 mL of sterile HS medium and 50 mL of yeast filtrate with different fermentation times were mixed, then *K. intermedius* was inoculated based on the ratio of 10^4 CFU/mL, and was incubated at 30°C for 7 d. Yeast filtrate with different fermentation times were added into 100 mL of HS culture medium, sterilised at 121°C for 20 min, inoculated with *K. intermedius* at the ratio of 10^4 CFU/mL, and incubated at 30°C for 7 d. After incubation, the BC was dried at 105°C until the quality was constant.

Activation, inoculation, and cultivation of yeast were as follows: malt extract medium was prepared by adding 2% wort extract, 2% glucose, 0.1% peptone, and 1.5% agar to deionised water. Yeast extract peptone dextrose (YPD) was prepared by adding 2% glucose, 2% peptone, and 1% yeast extract powder to deionised water, and the pH was about 5.0. The strains were transferred to wort agar medium to obtain single colonies, and then four rings of strains were inoculated into 50 mL of YPD medium, and incubated at 30°C for 24 h. The yeasts in the seed solution were counted by the dilution spread plate method. They were inoculated with *K. intermedius* into 100 mL of HS medium in different proportions after counting, and then cultured at 30°C for 7 d. After incubation, the BC was dried at 105°C until the quality was constant.

Activation, inoculation, and cultivation of kombucha were as follows: sugar tea was prepared by adding 1% (w/v) Dahongpao and 15% (w/v) glucose to deionised water, and the pH was about 4.0. Next, 40 g of kombucha biofilm (*K. intermedius* and yeast were inoculated into 250 mL of sugar tea water, and incubated at 30°C for 7 d, then inoculated with the new bacterial membrane produced by the liquid level) was added to 300 mL of sugar tea water, incubated at

30°C for 7 d, inoculated with the newly formed biofilm on the liquid surface, and 1 g of biofilm was added to 100 mL of HS medium (concentration of acetic acid bacteria was 10^4 CFU/mL, the concentration of yeast was 10^7 CFU/mL), and incubated at 30°C for 7 d. After incubation, the BC was dried at 105°C until the mass was constant.

Effect of yeast on BC yield

The concentration of acetic acid bacteria and yeast in 1 g of tea membrane was 10^4 and 10^7 CFU/mL. In order to investigate the effect of yeast on the synthesis of BC by *K. intermedius*, the inoculation amount of *K. intermedius* was fixed at 10^4 CFU/mL, and the inoculation amount of yeast was changed to increase the yield of BC after 7 d of static culture. BC produced by pure culture of acetic acid bacteria was used as control. The ratio of each factor was as follows:

1) The ratios of *K. intermedius* and *B. bruxellensis* were 1:1, 1:10, 1:100, and 1:1000 respectively.
2) The ratios of *K. intermedius* and *Z. bisporus* were 10:1, 1:1, 1:10, and 1:100, respectively.
3) The ratios of *K. intermedius* and *M. fructicola* were 10:1, 1:1, 1:10, and 1:100 respectively.

Optimum co-culture ratio of yeast and acetic acid bacteria.

Based on the results of single factor experiment, *B. bruxellensis* and *Z. bisporus* could promote the synthesis of BC by *K. intermedius*. *M. fructicola* had no effect on the synthesis of BC, so *M. fructicola* was not considered in the orthogonal experiment. A two-factor three-level full factor test was performed on *B. bruxellensis* and *Z. bisporus*, and the factor levels are shown in Table 1.

Table 1. Orthogonal test.

Level	Factor	
	***B. bruxellensis* (CFU/mL) A**	***Z. bisporus* (CFU/mL) B**
1	10^5	10^3
2	10^6	10^4
3	10^7	10^5

Based on the results of orthogonal test, *B. bruxellensis* (10^5 CFU/mL) and *Z. bisporus* (10^5 CFU/mL) were inoculated in 100 mL of HS medium, and incubated at 30°C for 1, 2, 3, 4, 5, 6, and 7 d. After fermentation, the fermented broth was centrifuged at 2,000 *g* in a MINI-6K centrifuge (Zhong Xing, Beijing, China) for 2 min to obtain yeast supernatant and thalli, and then the supernatant was filtered with a 0.22 μm filter membrane to remove residual thalli to obtain yeast filtrate, which was then stored at 4°C. The supernatant and centrifuged solid were used in fermentation assays.

Composition analysis of yeast filtrate

Determination of amino acids

Amino acids were determined by LA8080 amino acid automatic analyser (Hitachi Limited, Tokyo, Japan). Sample (5 mL) and 10 mL of 6 mol/L hydrochloric acid solution were added into the hydrolysis tube. The hydrolysis tube was then put into the refrigerant for freezing for 5 min, connected to the suction pipe of the vacuum pump, vacuumised (close to 0 Pa), filled with nitrogen, vacuumised again, again filled with nitrogen for three times, and the screw cover was tightened. The hydrolysis tube was hydrolysed for 22 h in an electric blast incubator at 110 ± 1°C, then taken out and cooled to room temperature. The hydrolysis tube was opened, and the hydrolysate was filtered into a 50 mL volumetric flask. The hydrolysis tube was washed with a small amount of water for many times, and the washing solution was transferred into the same 50 mL volumetric flask. Finally, the volume was fixed to the scale with water, and shaken evenly. Filtrate (1.0 mL) was then transferred into a 15 mL test tube, and dried under reduced pressure at 40°C. Next, 1.0 mL of sodium citrate buffer solution at pH 2.2 was dissolved again. After shaking and mixing, it was passed through 0.22 μm filter membrane. The injection volume of sulfonic acid cationic resin was 20 μL. The wavelengths were 570 and 440 nm. The flow rate of ninhydrin was 0.35 mL/min. The flow rate of mobile phase was 0.4 mL/min, and the reactor temperature was 135°C.

Determination of volatile substances

Volatile substances were determined by 7890A-5975C GC-MS (Agilent, California, America). Sample (5 mL) was put into a headspace bottle, and sealed with 1 g of sodium chloride. It was

extracted in a water bath at 50°C for 30 min. After extraction, they were desorbed at the injection port for 5 min. The Agilent hp-5ms chromatographic column was splitless. The temperature of the injection port was 250°C. The carrier gas was high purity Helium (99.999%). The flow rate was 1.0 mL/min. The starting temperature was 40°C, and it was maintained for 3 min. It was increased to 250°C at the rate of 5°C/min, and maintained for 8 min. The ion source temperature of electron ionisation was 230°C. The temperature of the four-stage rod was 180°C and full scanning.

Determination of organic acids

Organic acids were determined by 1200 HPLC (Agilent, California, America). Sample (5 mL) was placed in a 10 mL volumetric flask, and brought to the mark with mobile phase. It was ultrasonically extracted for 30 min, water-bathed at 60°C for 1 h, and filtered through 0.45 μm filter membrane. The column temperature of the Agilent SB-AQ C_{18} column was 30°C. The flow rate was 0.5 mL/min. The injection volume was 10 μL. The mobile phase was 10 mmoL K_2HPO_4 (pH 2.55), and the detection wavelength was 210 nm by a diode array detector.

Determination of vitamin B_1

Vitamin B_1 was determined by 1200 HPLC. Sample (2 mL) was placed in a test tube, and 1 mL of alkaline potassium ferricyanide was added. After vortexing and mixing, 2 mL of *n*-butanol was added. The treated samples were vortexed and mixed for 10 min after the layers were fully layered. The upper layer was drawn through a 0.45 μm filter membrane. The column temperature of SHISEIDO C_{18} column was 30°C. The flow rate was 1.0 mL/min. The injection volume was 10 μL. The ratio of mobile phase A (0.05 mol/L sodium acetate buffer) to B (methanol) was 65:35. The excitation wavelength of the fluorescence detector was 375 nm, and the detection wavelength was 435 nm.

Determination of vitamin B_2

Vitamin B_2 was determined by 1200 HPLC. Sample (5 mL) was diluted to 50 mL with water, and passed through 0.45 μm filter membrane. The column temperature of SHISEIDO C_{18} column was 30°C. The flow rate was 1.0 mL/min. The injection volume was 10 μL. The ratio of mobile phase A (0.05 mol/L sodium acetate buffer) to B (methanol) was 65:35. The excitation wavelength of the detector was 462 nm, and the detection wavelength was 522 nm.

Determination of vitamin B_6

Vitamin B_6 was determined by 1200 HPLC. Sample (5 mL) was placed in a 25 mL centrifuge tube, and an appropriate amount of water was added. It was extracted by ultrasonic for 30 min. The volume was adjusted to 50 mL, and passed through 0.45 μm filter membrane. The column temperature of SHISEIDO C_{18} column was 30°C. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. 50 mL of methanol, 2 g of sodium octane sulfonate, and 2.5 mL of triethylamine were dissolved in water, and the volume was adjusted to 1,000 mL. The pH was adjusted to 3.0 ± 0.1 with glacial acetic acid, and passed through 0.45 μm filter membrane. The excitation wavelength of the fluorescence detector was 293 nm, and the detection wavelength was 395 nm.

Determination of vitamin B_{12}

Vitamin B_{12} was determined by 1200 HPLC. Sample (5 mL) was placed in a 25 mL centrifuge tube, and an appropriate amount of water was added. It was ultrasonically extracted for 30 min, made up to 50 mL, and passed through 0.45 μm filter membrane. The column temperature of SHISEIDO C_{18} column was 25°C. The flow rate was 1.0 mL/min. The injection volume was 10 μL. The mobile phase A was acetonitrile, and B was 0.025% trifluoroacetic acid aqueous solution. The wavelength of the diode array detector was 361 nm.

Determination of vitamin C

Vitamin C was determined by 1200 HPLC. Sample (1 mL) was placed in a 10 mL volumetric flask, then 8 mL of 20 g/L metaphosphoric acid solution was added, and ultrasonically extracted for 30 min. The column temperature of SHISEIDO C_{18} column was 25°C. The flow rate was 0.8 mL/min. The injection volume was 10 μL. The mobile phase A was methanol, and 6.8 g of potassium dihydrogen phosphate and 0.91 g of cetyltrimethylammonium bromide were dissolved in water, then diluted to 1 L (adjusted to pH 2.5~2.8 with phosphoric acid) as B. Mobile phases A and B were mixed at a ratio of 2:98. It was then passed through 0.45 μm filter membrane, and degassed by ultrasonic. The wavelength of the diode array detector was 245 nm.

Statistical analysis

Results were presented as mean ± standard deviation of triplicate. Data were analysed statistically by One-way ANOVA and One-way dependent variable ANOVA using IBM SPSS Statistics 23 software. Probability values of $p < 0.05$ were considered significantly different.

Results and discussion

Effect of yeast on BC yield of K. intermedius

The yield of BC co-cultured by *K. intermedius* and *B. bruxellensis* in different proportions are shown in Figure 1a. It was apparent that with the increase in the proportion of *B. bruxellensis*, the yield of BC first increased, and then decreased. But both were significantly higher than those of the control group ($p < 0.05$), thus indicating that *B. bruxellensis* could promote the synthesis of BC by *K. intermedius*. Tran *et al.* (2022) showed that *B. bruxellensis* was the dominant fungal species during the fermentation of kombucha. With the prolongation of fermentation time, the weight of BC increased significantly from 1.71 ± 0.4 to 11.8 ± 5.4 mg. When there were acetic acid bacteria, *B. bruxellensis* grew slightly better. Studies by Villarreal-Soto *et al.* (2020) showed that kombucha samples had high proportion of *Schizosaccharomyces pombe* and *B. bruxellensis* in the liquid phase, which had the fastest sugar consumption rate and highest ethanol content. When the ratio of *K. intermedius* to *B. bruxellensis* was 1:100 (*B. bruxellensis* was 10^6 CFU/mL), the highest yield of BC was 4.70 g/L, which was about 3.2 times that of pure cultures of *K. intermedius*, but still less than that of kombucha.

The yield of BC co-cultured by *K. intermedius* and *Z. bisporus* in different proportions are shown in Figure 1b. With the increase in the proportion of *Z. bisporus*, the yield of BC first increased, and then decreased. But both were significantly higher than those of the control group ($p < 0.05$), thus indicating that *Z. bisporus* could promote *K. intermedius* to synthesise BC. During fermentation, *Z. bisporus* utilised sucrose to produce glucose and fructose, and underwent alcoholic fermentation to produce ethanol (Abaci *et al.*, 2022). With the increase in the proportion of *Z. bisporus*, the ethanol content in the solution increased. When the ethanol reaches a certain concentration, it might inhibit the growth and metabolism of acetic acid bacteria, so the BC showed a trend of first increasing and then decreasing (Vashisht *et al.*, 2019). When the ratio of *K.*

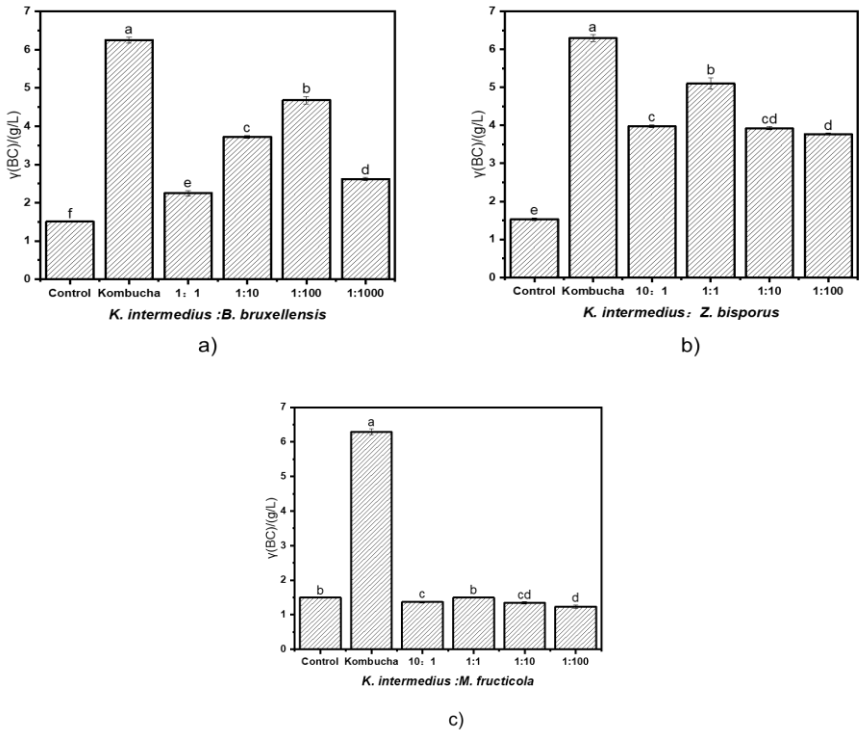

Figure 1. (a) BC produced by *K. intermedius* co-cultured with *B. bruxellensis*. **(b)** BC produced by *K. intermedius* co-cultured with *Z. bisporus*. **(c)** BC produced by *K. intermedius* co-cultured with *M. fructicola*.

intermedius to *Z. bisporus* was 1:1 (*Z. bisporus* was 10^4 CFU/mL), the highest yield of BC was 5.19 g/L, which was about 3.5 times that of pure culture of *K. intermedius*. But it was still slightly less than that of kombucha.

The yield of BC co-cultured by *K. intermedius* and *M. fructicola* in different proportions are shown in Figure 1c. With the increase in the proportions of *M. fructicola*, the yield of BC first increased, and then decreased, and it was significantly lower than that of kombucha ($p < 0.05$). There was no significant difference between the yield of BC under different proportions and the control group ($p > 0.05$), thus indicating that *M. fructicola* could hardly promote the synthesis of BC by *K. intermedius*. This might be related to the superoxide anion and H_2O_2 produced during the growth and reproduction of *M. fructicola* (Abaci *et al.*, 2022). The yield of BC in different proportions was equal, and had little relationship to the quantity of *M. fructicola*.

To sum up, *B. bruxellensis* and *Z. bisporus* could promote the synthesis of BC by *K. intermedius*, while *M. fructicola* had no effect on the synthesis of BC. In the co-culture process of *K. intermedius* and *B. bruxellensis*, when *K. intermedius*:*B. bruxellensis* was 1:100, the highest yield of BC was 4.70 g/L. In the co-culture process of *K. intermedius* and *Z. bisporus*, when *K. intermedius*:*Z. bisporus* was 1:1, the highest yield of BC was 5.19 g/L. However, the yield of BC of kombucha was still slightly higher than that of *K. intermedius* co-cultured with *B. bruxellensis* or *Z. bisporus*. The microbial composition of kombucha community is complex. The unseparated microorganisms may also promote the synthesis of BC, but the details are not clear.

Orthogonal test of B. bruxellensis and Z. bisporus

Based on the results of single factor test, *M. fructicola* had no obvious promotion effect on BC synthesis ($p > 0.05$). So, *M. fructicola* was not considered in orthogonal test. The yield of BC was the highest when the inoculation amount of *B. bruxellensis* was 10^6 CFU/mL, and that of *Z. bisporus* was 10^4 CFU/mL. The optimal level of *B. bruxellensis* was selected by one level above and below, namely 10^5 and 10^7 CFU/mL. The optimal level of *Z. bisporus* was selected by one level above and below, namely 10^3 and 10^5 CFU/mL. A two-factor three-level full factorial test was performed, and the results are shown in Table 2. When the

Table 2. Orthogonal test results of *B. bruxellensis* and *Z. bisporus*.

Number	Factor		Yield of BC
	A	B	(g/L)
1	1	1	4.847
2	1	2	4.960
3	1	3	5.515
4	2	1	4.661
5	2	2	4.726
6	2	3	4.743
7	3	1	3.813
8	3	2	3.844
9	3	3	3.994
K_1	15.322	13.321	
K_2	14.130	13.530	
K_3	11.651	14.252	
$\bar{K}_1$	5.107	4.440	
$\bar{K}_2$	4.710	4.510	
$\bar{K}_3$	3.884	4.751	
Optimal levels	A_1	B_3	
R (range)	1.223	0.311	
Important order	AB		

A: *B. bruxellensis*; B: *Z. bisporus*; 1, 2, 3: three levels of these factors are shown in Table 2; K: sum of factor experiment results; and $\bar{K}$: average value of K.

inoculation amount of *B. bruxellensis* was 10^5 CFU/mL and the inoculation amount of *Z. bisporus* was 10^5 CFU/mL, the BC yield was the highest, which was 5.51 g/L.

The range calculations (Table 2) showed that the optimal level combination of relevant factors was A1B3, and the order of importance of the two factors was *B. bruxellensis* and *Z. bisporus*.

Effects of yeast filtrate in different fermentation times and inactivated yeast on BC yield.

To determine how *B. bruxellensis* and *Z. bisporus* promoted the synthesis of BC by *K. intermedius*, yeast fermentation broth fermented for different times was separated into yeast filtrate and body by centrifugation, which were later added to HS culture medium to clarify the way in which yeast promoted the synthesis of BC by acetic acid bacteria. The yield of BC was determined after standing for 7 d.

The yield of BC after adding yeast filtrate with different fermentation times are shown in Figure 2a. The yield of BC after adding yeast filtrate was significantly higher than that of the control group without adding yeast filtrate ($p < 0.05$). The yeast filtrate in the early stage of fermentation (1~3 days) had greater influence on the yield of BC, and the growth amount of BC was greater. While the yeast filtrate in the late stage of fermentation (4~7 days) had little influence on the growth amount of BC. The yield of BC after adding yeast filtrate for 7 d was the highest, which was 5.42 g/L, about 3.7 times of the control group without adding yeast filtrate, and slightly lower than the orthogonal test results, which indicated that the metabolites of *B. bruxellensis* and *Z. bisporus* could promote the synthesis of BC. Devanthi *et al.* (2021) showed that with the progress of fermentation, the total number of colonies continued to increase, and yeast and acetic acid bacteria grew together, which might be related to metabolites, such as amino acids, vitamins, ethanol, *etc*. Moreover, *B. bruxellensis* had higher consumption rate of reducing sugar, which was much higher than that of *K. intermedius*. Therefore, the growth and metabolism of yeast could promote the growth and reproduction of acetic acid bacteria, and accelerate the synthesis of BC. Kim *et al.* (1996) showed that the addition of yeast extract greatly stimulated the production of BC. When the concentration of yeast extract was 8 g/L, the highest yield of BC (4.49 g/L) was obtained.

The yield of BC after adding inactivated yeasts with different fermentation times are shown in Figure 2b. The yield of BC after adding inactivated yeast was higher than that of the control group without adding inactivated yeast significantly ($p < 0.05$), and the inactivated yeast with different fermentation times had little effect on the yield of BC. After adding inactivated yeast for 7 d, the yield of BC was the highest, which was 2.89 g/L, which was about 2.0 times that of the control group, which indicated that inactivated yeast could promote BC synthesis to a certain extent. Yeast contains a variety of nutrients, including proteins, carbohydrates, RNA, glutathione, vitamin B, *etc*. (Lin *et al.*, 2014). These nutrients will be released after yeast rupture, which may promote the synthesis of BC, but the details are not clear.

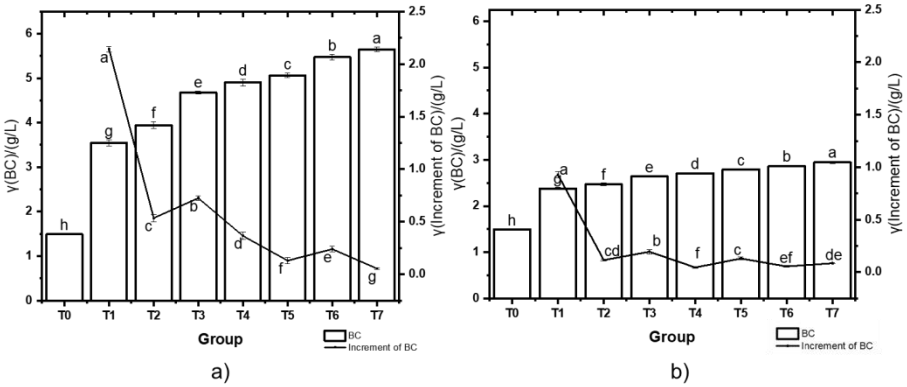

Figure 2. (a) Effect of medium with yeast fermentation supernatant of different fermentation times on BC production of *K. intermedius* (Tn represents the fermentation time of yeast supernatant). **(b)** Effect of medium with inactivated yeast cells of different fermentation times on BC production of *K. intermedius* (Tn represents the fermentation time of yeast cells).

To sum up, the metabolites of yeast could promote the synthesis of BC by *K. intermedius*, and the yeast filtrate in the early stage of fermentation (1~3 d) had greater impact on the yield of BC, and the growth amount was greater. Inactivated yeast could also promote the production of BC.

Composition analysis of yeast filtrate

Amino acids

Yeast can secrete a variety of amino acids into the culture medium during its growth. The content of 17 amino acids in the unfermented HS culture medium and yeast filtrate was detected by the amino acid analyser. The results are shown in Table 3.

It could be seen from Table 3 that 15 kinds of amino acids were detected in unfermented HS medium, with a total content of 1.465 g/L, and 16 kinds of amino acids were detected in yeast filtrate, cysteine appeared, with a total content of 2.323 g/L, which increased by 59.68% when compared with the unfermented HS medium. After yeast fermentation, the biggest change in the content of 17 amino acids was isoleucine, which was 11.64 times higher than that before fermentation; aspartic acid increased by 60.00%, ranked second; glycerine increased by 41.67%, ranked third. While tyrosine and phenylalanine decreased by 38.57 and 31.67%, respectively. Zhao *et al.* (2018) also found that when compared with tea syrup, the total amount of free amino acids in the fermentation broth of kombucha increased. The increase in amino acids might be related to the autolysis produced by yeast. The reduction of amino acids might be related to the formation of different compounds.

Table 3. Contents of 17 amino acids.

Amino acid content	Unfermented HS medium (g/L)	Yeast filtrate (g/L)
Aspartic acid	0.102 ± 0.002^{e}	0.161 ± 0.002^{e}
Threonine	0.053 ± 0.004^{g}	0.068 ± 0.000^{n}
Serine	0.053 ± 0.002^{g}	0.072 ± 0.003^{j}
Glutamate	0.172 ± 0.004^{a}	0.230 ± 0.000^{b}
Glycine	0.122 ± 0.006^{d}	0.170 ± 0.002^{d}
Alanine	0.152 ± 0.004^{b}	0.190 ± 0.002^{c}
Cysteine	nd	0.015 ± 0.001^{m}
Valine	0.085 ± 0.005^{f}	0.101 ± 0.003^{h}
Methionine	nd	nd
Isoleucine	0.061 ± 0.004^{g}	0.709 ± 0.003^{a}
Leucine	0.121 ± 0.003^{d}	0.121 ± 0.003^{f}
Tyrosine	0.139 ± 0.003^{c}	0.087 ± 0.002^{i}
Phenylalanine	0.121 ± 0.002^{d}	0.082 ± 0.001^{i}
Lysine	0.101 ± 0.004^{e}	0.111 ± 0.004^{g}
Histidine	0.028 ± 0.004^{h}	0.035 ± 0.002^{l}
Arginine	0.059 ± 0.002^{g}	0.061 ± 0.002^{k}
Proline	0.096 ± 0.006^{e}	0.110 ± 0.002^{g}

nd: not detected.

Volatile substance

Yeast can produce a variety of volatile substances such as alcohols and esters during its growth. The volatile substances in the unfermented HS medium and yeast filtrate were analysed by GC-MS. The volatile components are shown in Table 4.

A total of 43 volatile substances were detected in unfermented HS medium, included five alcohols, eight aldehydes, seven acids, four alkenes, three ketones, five aromatic compounds, three esters, five heterocyclic compounds, and three others, with relative contents of 61.85, 6.77, 8.62, 1.29, and

Table 4. Volatile substances of unfermented HS medium and yeast filtrate.

Serial number	Unfermented HS medium		Yeast filtrate	
	Compound name	Relative content (%)	Compound name	Relative content (%)
	Alcohol			
1	Ethyl alcohol	59.83	Ethyl alcohol	77.35
2	2-Ethylhexanol	0.22	3-Methyl-1-butanol	4.13
3	Phenylethyl alcohol	1.32	2-Ethylhexanol	0.07
4	cis-3-Methylpent-3-ene-5-ol	0.28	Phenylethyl alcohol	3.14
5	2(R),3(S)-1,2,3,4-Butanetetrol	0.20	2-(4-Methyl-3-cyclohexenyl)-2-propanol	0.06
6			Tetramethyl-5-decyne-4,7-diol	0.08
7			3,6,6-Trimethylbicyclo[3.1.1]heptan-2-ol	0.06
	Aldehyde			
1	3-Methylthiopropionaldehyde	0.18	Benzaldehyde	0.38
2	Benzaldehyde	0.77	Octanal	0.14
3	Benzeneacetaldehyde	1.13	Benzeneacetaldehyde	0.12
4	2-Hydroxy-4-methylbenzaldehyde	0.26	Nonanal	0.43
5	Nonanal	0.95	Decanal	0.05
6	Decanal	0.22	3,5-Dimethylbenzaldehyde	0.65
7	3,5-Dimethylbenzaldehyde	3.00	Acetaldehyde	0.05
8	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	0.26	3,5-di-tert-Butyl-4-hydroxybenzaldehyde	0.08
	Acid			
1	Hexanoic acid	0.15	Octanoic acid	0.43
2	Octanoic acid	1.75	Nonanoic acid	0.09
3	Nonanoic acid	0.50	*n*-Decanoic acid	0.21
4	Undecylenic acid	1.13	Pentadecanoic acid	0.05
5	*n*-Decanoic acid	4.22	*n*-Hexadecanoic acid	0.06
6	Dodecanoic acid	0.32		
7	*n*-Hexadecanoic acid	0.53		
	Alkane			
1	1-Dodecene	0.71	(E)-3-Hexene	0.05
2	Isobutyl cyclopentane	0.19	1-Tetradecene	0.14
3	Eicosane	0.22		
4	(Z,Z)-α-Farnesene	0.16		
	Ketone			
1	Tetrahydro-3,6-dimethyl-2H-pyran-2-one	0.43		
2	2-Methyl-4-heptanone	0.19		
3	7,9-di-tert-Butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	0.42		
	Aromatic compound			
1	1,2,4,5-Tetratoluene	0.31	3-Methylphenol	0.06
2	1,3-Diethylbenzene	0.19	1,2,4,5-Tetratoluene	0.08
3	Naphthalene	0.15	Naphthalene	0.05
4	2,4-di-tert-Butylphenol	12.84	2,4-di-tert-Butylphenol	8.59
5	*p*-Nitrostyrene	0.27	3-Nitrostyrene	0.10
	Ester			
1	Sulphurous acid, decylhexyl ester	0.16	DL-Tyrosine ethyl ester	0.07
2	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.85	(2,4-Dimethylphenyl)methyl 2,5-dimethylbenzoate	0.05
3	Dibutyl phthalate	1.46	Acetic acid, 2-phenylethyl ester	0.07
4			Propanoic acid, 2-methylbutyl ester	0.05

5			Oxalic acid, decylneopentyl ester	0.10
6			Methyl 10-methyldodecanoate	0.16
7			Phthalic acid, 4-fluoro-2-nitrophenyl-2-pentyl ester	0.34
8			Dibutyl phthalate	0.76
		Heterocyclic compound		
1	2,5-Dimethylpyrazine	0.50	7-Methyl-7H-dibenzocarbazole	0.06
2	7-Methyl-7H-dibenzocarbazole	0.18	Tetrahydrothiazole	0.15
3	2-Acetylthiazole	0.18	Indole	0.12
4	Indole	1.14	3-Methylindole	0.28
5	5-Methylindole	1.07		
		Other		
1	2,6-di-tert-Butylbenzenone	0.44	Phenethylamine, N-methyl-β-3,4-tris(trimethylsiloxy)	0.29
2	2,4-bis[(Trimethylsilyl)oxy]trimethylsilyl benzoate	0.42	Methionine	0.08
3	3-Trimethylsilyloxystearic acid, trimethylsilyl ester	0.27	2,6-di-tert-Butylbenzoquinone	0.16
4			1,2-Phenylenebis(Dimethylarsine)	0.05
5			Benzoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	0.30
6			5-Methyl-1,2,4-triazolo[4,3-a]pyridine-3-thiol	0.16
7			1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-Octasiloxane	0.05

1.04%, respectively. A total of 46 volatile substances were detected in yeast filtrate, including seven alcohols, eight aldehydes, five acids, two alkenes, five aromatic compounds, eight esters, four heterocyclic compounds, and seven others. No ketones were detected, with relative contents of 84.89, 1.91, 0.84, 0.19, 8.88, 1.59, 0.61, and 1.09%, respectively.

The relative content of alcohols in the unfermented HS medium and yeast filtrate were the highest, and all of them contained ethanol, 2-ethylhexanol, and phenylethanol. After yeast fermentation, the relative content of alcohols increased by 23.04%, and 3-methyl-1-butanol, 2-(4-methyl-3-cyclohexenyl)-2-propanol, tetramethyl-5-design-4,7-dial, and 3,6,6-trimethylbicyclo were produced. The relative content of ethanol in yeast filtrate was 77.35%, with the largest increase of 17.52%. The relative content of 3-methyl -1-butanol was 4.13%, second only to ethanol, and the content of phenylethanol were 3.14%, which increased by 1.82% when compared with unfermented HS medium. Alcohols give the product an alcoholic taste. The production of alcohols is directly related to the metabolism of amino acids, such as leucine, isoleucine, valine, *etc*. These amino acids are catabolised to form higher alcohols. Aldehydes are reduced to higher alcohols by the Ehrlich pathway. Higher alcohols have a positive effect on human body (Chakravorty *et al.*, 2016). The relative content of aromatic compounds was second only to alcohols. The common compounds in the unfermented HS medium and yeast filtrate included 2,4-di-tert-butylphenol, 1,2,4,5-tetramethylbenzene, and naphthalene. After yeast fermentation, new compounds such as 3-nitroethylene and 3-methylphenol were produced, but the total relative content of aromatic compounds decreased by 4.88%. The relative content of acids ranked third. After yeast fermentation, the species decreased from seven to five, and the relative content also decreased by 7.78%. Octanoic acid accounted for the largest proportion of acids. There were eight aldehydes in the unfermented HS medium and yeast filtrate, but the relative content in yeast filtrate decreased by 4.86%. Acids give the product a sour taste. Although the number of esters increased from three to eight, the relative content decreased by 0.88%. Under the action of esterase, alcohols and acids can be dehydrated and condensed to form esters. The formation of esters is also related to the intracellular mediation of yeast. Although there are trace amounts of esters, they have an important impact on aroma. In addition, alkylenes decreased by 1.1%, heterocyclic compounds decreased by 2.46%, and others decreased by 0.04%.

Generally speaking, when compared with unfermented HS medium, the relative content of alcohols in yeast filtrate increased greatly, while the relative content of other substances decreased in different degrees. It was different from the acid content increase measured by Zhao *et al.* (2018) in the study on the preparation of black tea fungus from pu'er tea. This was due to the different strains used, which contain six kinds of bacteria.

Organic acid

The contents of oxalic, tartaric, malic, lactic, acetic, citric, succinic, maleic, and fumaric acids in the unfermented HS medium and yeast filtrate were detected by HPLC. The contents of nine organic acids in unfermented HS medium and yeast filtrate are shown in Table 5.

Table 5. Contents of nine organic acids.

Organic acid	Unfermented HS medium (mg/L)	Yeast filtrate (mg/L)
Oxalic acid	nd	nd
Tartaric acid	6.52 ± 0.03[c]	2.19 ± 0.02[c]
Malic acid	nd	nd
Lactic acid	nd	nd
Acetic acid	nd	nd
Citric acid	46.53 ± 0.02[a]	39.77 ± 0.03[a]
Succinic acid	37.03 ± 0.02[b]	16.06 ± 0.04[b]
Maleic acid	nd	nd
Fumaric acid	nd	nd

nd: not detected.

Three kinds of organic acids namely tartaric, citric, and succinic acids were detected in unfermented HS medium, and their contents were 6.52, 46.53, and 37.03 mg/L, respectively. Only tartaric, citric, and succinic acids were detected in yeast filtrate, and their contents were 2.19, 39.77, and 16.06 mg/L, respectively. When compared with the unfermented HS medium, they were reduced by 66.36, 14.53, and 56.63%, respectively. Torija *et al.* (2003) studied the fermentation rate of commercial *Saccharomyces cerevisiae* in the medium containing different concentrations of tartaric acid. The results showed that when the concentration of tartaric acid was 5 and 10 g/L, the fermentation rate was the highest, and when the medium did not contain tartaric acid, the fermentation stopped. During the fermentation process, tartaric acid accumulated in cells, and reached the highest content at the end of fermentation. This might be one of the reasons for the decrease in tartaric acid content, which was consistent with the decrease in tartaric acid content observed in the present work. Citric acid and succinic acid are the same. Although some studies had found that there are many kinds of organic acids in the fermentation broth of kombucha, the types of organic acids in HS culture medium after yeast fermentation did not increase, and the content of organic acids decreased (Jayabalan *et al.*, 2007; Villarreal-Soto *et al.*, 2018).

Vitamins

Vitamin B_1, B_2, B_6, B_{12}, and C in the unfermented HS medium and yeast filtrate were detected by HPLC. The results are shown in Table 6.

Table 6. Content of five vitamins.

Name		Unfermented HS medium (mg/L)	Yeast filtrate (mg/L)
Vitamin B_1		nd	nd
Vitamin B_2		0.200 ± 0.001[c]	0.201 ± 0.001[c]
Vitamin B_6	Pyridoxal	1.012 ± 0.001[b]	0.976 ± 001[b]
	Pyridoxine	nd	nd
	Pyridoxamine	11.064 ± 0.006[a]	10.750 ± 0.007[a]
Vitamin B_{12}		nd	nd
Vitamin C		nd	nd

nd: not detected.

Vitamins B_2 and B_6 were detected in unfermented HS medium, the contents of which were 0.200 and 12.076 mg/L, respectively. Only vitamins B_2 and B_6 were detected in yeast filtrate. The content of vitamin B_2 was the same as that of unfermented HS medium, and the content of vitamin B_6 was slightly lower than that of unfermented HS medium, which was 11.726 mg/L. Bauer-Petrovska and Petrushevska-Tozi (2010) found that vitamins B_1, B_6, B_{12} and C in tea syrup increased by 61, 83, 131, and 114%, respectively. However, the types and contents of vitamins did not change obviously after HS medium was fermented by yeast.

Conclusion

In the previous work, one strain of acetic acid bacteria (*K. intermedius*) and three strains of yeasts (*B. bruxellensis*, *Z. bisporus*, and *M. fructicola*) were isolated from the commercial kombucha in China. In order to clarify the mechanism of yeasts promoting the synthesis of bacterial cellulose by acetic acid bacteria, *B. bruxellensis*, *Z. bisporus*, and *M. fructicola* were co-cultured with *K. intermedius* in different proportions. Both *B. bruxellensis* and *Z. bisporus* could promote BC synthesis, with the highest yields of 4.70 and 5.19 g/L, respectively. *M. fructicola* had no obvious effect on BC synthesis. Based on the orthogonal test, the optimal co-culture ratio of *K. intermedius*, *B. bruxellensis*, and *Z. bisporus* was 1: 10: 10, and the yield of BC was 5.51 g/L. Yeast filtrate and inactivated yeast were added to *K. intermedius* culture medium respectively. Yeast filtrate had strong promotion effect on BC synthesis, while yeast had little effect on BC yield, which indicated that yeast metabolites could promote BC synthesis. Amino acids, volatile substances, organic acids, and vitamins in yeast filtrate were detected. After yeast fermentation, the variety and total amount of amino acids increased, cysteine was newly formed, and the contents of isoleucine, aspartic acid, and glycerine increased significantly. The relative content of ethanol increased significantly, and the relative content of 3-methyl-1-butanol and phenylethanol also increased significantly. The contents of organic acids and vitamins had no obvious change. The present work focused on the interaction between yeast and acetic acid bacteria in black tea fungus, and how yeast could promote acetic acid bacteria to produce BC. The results showed that yeast stimulated the growth of acetic acid bacteria and promoted the production of BC by acetic acid bacteria. Yeast filtrate could promote the production of BC more than yeast cell. The type and content of amino acids in yeast filtrate, and the content of some volatile substances increased when compared with those before fermentation, and the nutrients provided to acetic acid bacteria were more abundant. The present work provided a theoretical basis for improving the production of BC, and also provided a reference for studying the interaction between species in the microbial community.

The interaction relationship between one strain of acetic acid bacteria (*K. intermedius*) and three strains of yeast (*B. bruxellensis*, *Z. bisporus*, and *M. fructicola*) in a commercial kombucha sample were described, but the overall situation in the sample was not explained. Therefore, further studies are still needed to elucidate the mechanism of yeast in the sample that promotes the synthesis of BC by acetic acid bacteria.

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References

Abaci, N., Deniz, F. S. S. and Orhan, I. E. 2022. Kombucha - An ancient fermented beverage with desired bioactivities: A narrowed review. Food Chemistry X 14: 100302.

Avcioglu, N. H., Birben, M. and Seyis Bilkay, I. 2021. Optimization and physicochemical characterization of enhanced microbial cellulose production with a new kombucha consortium. Process Biochemistry 108: 60-68.

Bauer-Petrovska, B. and Petrushevska-Tozi, L. 2010. Mineral and water soluble vitamin content in the kombucha drink. International Journal of Food Science and Technology 35(2):201-205.

Bose, S., Roy, M. and Bandyopadhyay, A. 2012. Recent advances in bone tissue engineering

scaffolds. Trends in Biotechnology 30(10): 546-554.

Chakravorty, S., Bhattacharya, S., Chatzinotas, A., Chakraborty, W., Bhattacharya, D. and Gachhui, R. 2016. Kombucha tea fermentation: Microbial and biochemical dynamics. International Journal of Food Microbiology 220: 63-72.

Coton, M., Pawtowski, A., Taminiau, B., Burgaud, G. Daniel, F., Coulloumme-Labarthe, L., … and Coton, E. 2017. Unraveling microbial ecology of industrial-scale kombucha fermentations by metabarcoding and culture-based methods. FEMS Microbiology Ecology 93(5): fix048.

Devanthi, P. V. P., Kho, K., Nurdiansyah, R., Briot, A., Taherzadeh, M. J. and Aslanzadeh, S. 2021. Do kombucha symbiotic cultures of bacteria and yeast affect bacterial cellulose yield in molasses? Journal of Fungi 7: 705.

Emiljanowicz, K. E. and Malinowska-Pańczyk, E. 2020. Kombucha from alternative raw materials – The review. Critical Reviews in Food Science and Nutrition 60(19): 3185-3194.

Gayathri, G. and Srinikethan, G. 2019. Bacterial cellulose production by *K. saccharivorans* BC1 strain using crude distillery effluent as cheap and cost effective nutrient medium. International Journal of Biological Macromolecules 138: 950-957.

Jakubczyk, K., Kałduńska, J., Kochman, J. and Janda, K. 2020. Chemical profile and antioxidant activity of the kombucha beverage derived from white, green, black and red tea. Antioxidants 9(5): 447.

Jayabalan, R., Malbaša, R. V., Lončar, E. S., Vitas, J. S. and Sathishkumar, M. 2014. A review on kombucha tea—Microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Comprehensive Reviews in Food Science and Food Safety 13(4): 538-550.

Jayabalan, R., Marimuthu, S. and Swaminathan, K. 2007. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. Food Chemistry 102(1): 392-398.

Jozala, A. F., de Lencastre-Novaes, L. C., Lopes, A. M., de Carvalho Santos-Ebinuma, V., Mazzola, P. G., Pessoa-Jr, A., … and Chaud, M. V. 2016. Bacterial nanocellulose production and application: A 10-year overview. Applied Microbiology and Biotechnology 100(5): 2063-2072.

Kim, J., Alizadeh, P., Harding, T., Hefner-Gravink, A. and Klionsky, D. J. 1996. Disruption of the yeast ATH1 gene confers better survival after dehydration, freezing, and ethanol shock: potential commercial applications. Applied and Environmental Microbiology 62(5): 1563-1569.

Krasteva, P. V., Bernal-Bayard, J., Travier, L., Martin, F. A., Kaminski, P.-A., Karimova, G., … and Ghigo, J.-M. 2017. Insights into the structure and assembly of a bacterial cellulose secretion system. Nature Communications 8(1): 2065.

Lin, D., Lopez-Sanchez, P., Li, R. and Li, Z. 2014. Production of bacterial cellulose by *Gluconacetobacter hansenii* CGMCC 3917 using only waste beer yeast as nutrient source. Bioresource Technology 151: 113-119.

Martínez Leal, J., Valenzuela Suárez, L., Jayabalan, R., Huerta Oros, J. and Escalante-Aburto, A. 2018. A review on health benefits of kombucha nutritional compounds and metabolites. CyTA - Journal of Food 16(1): 390-399.

Nguyen, N. K., Nguyen, P. B., Nguyen, H. T. and Le, P. H. 2015. Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid. LWT - Food Science and Technology 64(2): 1149-1155.

Ponomarova, O., Gabrielli, N., Sévin, D. C., Mülleder, M., Zirngibl, K., Bulyha, K., … and Patil, K. R. 2017. Yeast creates a niche for symbiotic lactic acid bacteria through nitrogen overflow. Cell Systems 5(4): 345-357.

Rahmani, R., Beaufort, S., Villarreal-Soto, S. A., Taillandier, P., Bouajila, J. and Debouba, M. 2019. Kombucha fermentation of African mustard (*Brassica tournefortii*) leaves: Chemical composition and bioactivity. Food Bioscience 30: 100414.

Rajwade, J. M., Paknikar, K. M. and Kumbhar, J. V. 2015. Applications of bacterial cellulose and its composites in biomedicine. Applied Microbiology and Biotechnology 99(6): 2491-2511.

Reiniati, I., Hrymak, A. N. and Margaritis, A. 2017. Recent developments in the production and applications of bacterial cellulose fibers and

nanocrystals. Critical Reviews in Biotechnology 37(4): 510-524.

Ross, P., Mayer, R. and Benziman, M. 1991. Cellulose biosynthesis and function in bacteria. Microbiological Reviews 55(1): 35-58.

Shahbazi, H., Hashemi Gahruie, H., Golmakani, M.-T., Eskandari, M. H. and Movahedi, M. 2018. Effect of medicinal plant type and concentration on physicochemical, antioxidant, antimicrobial, and sensorial properties of kombucha. Food Science and Nutrition 6(8): 2568-2577.

Sharma, C. and Bhardwaj, N. K. 2019. Biotransformation of fermented black tea into bacterial nanocellulose *via* symbiotic interplay of microorganisms. International Journal of Biological Macromolecules 132: 166-177.

Stadie, J., Gulitz, A., Ehrmann, M. A. and Vogel, R. F. 2013. Metabolic activity and symbiotic interactions of lactic acid bacteria and yeasts isolated from water kefir. Food Microbiology 35(2): 92-98.

Subbiahdoss, G., Osmen, S. and Reimhult, E. 2022. Cellulosic biofilm formation of *Komagataeibacter* in kombucha at oil-water interfaces. Biofilm 4: 100071.

Tian, D., Shen, F., Hu, J., Renneckar, S. and Saddler, J. N. 2018. Enhancing bacterial cellulose production *via* adding mesoporous halloysite nanotubes in the culture medium. Carbohydrate Polymers 198: 191-196.

Torija, M. J., Rozès, N., Poblet, M., Guillamón, J. M. and Mas, A. 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. International Journal of Food Microbiology 80(1): 47-53.

Tran, T., Verdier, F., Martin, A., Alexandre, H., Grandvalet, C. and Tourdot-Maréchal, R. 2022. Oxygen management during kombucha production: Roles of the matrix, microbial activity, and process parameters. Food Microbiology 105: 104024.

Vashisht, A., Thakur, K., Kauldhar, B. S., Kumar, V. and Yadav, S. K. 2019. Waste valorization: Identification of an ethanol tolerant bacterium *Acetobacter pasteurianus* SKYAA25 for acetic acid production from apple pomace. Science of the Total Environment 690: 956-964.

Villarreal-Soto, S. A., Beaufort, S., Bouajila, J., Souchard, J.-P. and Taillandier, P. 2018. Understanding kombucha tea fermentation: A review. Journal of Food Science 83(3): 580-588.

Villarreal-Soto, S. A., Bouajila, J., Pace, M., Leech, J., Cotter, P. D., Souchard, J.-P., … and Beaufort, S. 2020. Metabolome-microbiome signatures in the fermented beverage, Kombucha. International Journal of Food Microbiology 333: 108778.

Zhao, Z. J., Sui, Y. C., Wu, H. W., Zhou, C. B., Hu, X. C. and Zhang, J. 2018. Flavour chemical dynamics during fermentation of kombucha tea. Emirates Journal of Food and Agriculture 30(9): 732-741.

Zhou, D.-D., Saimaiti, A., Luo, M., Huang, S.-Y., Xiong, R.-G., Shang, A., … and Li, H.-B. 2022. Fermentation with tea residues enhances antioxidant activities and polyphenol contents in kombucha beverages. Antioxidants 11(1): 155.

Zou, C., Li, R.-Y., Chen, J.-X., Wang, F., Gao, Y., Fu, Y.-Q., … and Yin, J.-F. 2021. Zijuan tea-based kombucha: Physicochemical, sensorial, and antioxidant profile. Food Chemistry 363: 130322.

Zoumpourtikoudi, V., Pyrgelis, N., Chatzigrigoriou, M., Tasakis, R. N. and Touraki, M. 2018. Interactions among yeast and probiotic bacteria enhance probiotic properties and metabolism offering augmented protection to *Artemia franciscana* against *Vibrio anguillarum*. Microbial Pathogenesis 125: 497-506.