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Screening the optimal ratio of symbiosis between isolated yeast and acetic acid bacteria strain from traditional kombucha for high-level production of glucuronic acid

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ABSTRACT

Glucuronic acid-a human detoxifying drug can be found in traditional kombucha which is a sweetened-black tea fermented by symbiotic microflora between yeast and acetic acid bacteria embedded within a microbial cellulose membrane. The main purpose of the study is to obtain the new designed symbiosis from the isolated yeasts and bacterial strains which can produce the high-level glucuronic acid kombucha and avoid unexpected microbial contaminants. The isolation, selection and identification showed the best initial combination ratio between *Dekkera bruxellensis* KN89 and *Gluconacetobacter intermedius* KN89 is 4Y (yeast):6A (acetic acid -bacteria) in number of living cell per milliliter which produced 175.8 mg L^{-1} glucuronic acid in 7-day fermentation ($P < 0.05$). This study also provides a basic understanding about fermentation kinetics of this symbiosis in order to control and enhance the final product at the critical time point (after 54 hrs of process). The findings of this study are practically relevant in producing a safe and glucuronic acid enriched kombucha.

1. Introduction

Kombucha or “tea fungus” is a healthy sugared tea, which is fermented by a symbiosis of acidophilic yeast and acetic acid bacteria (AAB) embedded in a microbial cellulose layer (Greenwalt, Steinkraus, & Ledford, 2000). One of the most significant organic compounds found in this drink is glucuronic acid (GlcUA) (Jayabalan, Malbasa, Loncar, Vitas, & Sathishkumar, 2014). This acid has been a topic of interest in recent years, because of its detoxifying properties. It can eliminate many types of toxicants such as pollutants, exogenous chemicals, excess steroid hormones, and bilirubin from the human body via the urinary system (Vīna, Linde, Patetko, & Semjonovs, 2013; Vīna, Semjonovs, Linde, & Patetko, 2013). Moreover, GlcUA can be converted into glucosamine, a beneficial substance associated with cartilage, collagen, and fluids related to the treatment of osteoarthritis (Yavari, Assadi, Moghadam, & Larijani, 2011). GlcUA is also a precursor for the biosynthesis of vitamin C (Merchie, Lavens, & Sorgeloos, 1997). In kombucha, AAB assimilate the monosaccharide (glucose and fructose) products of yeast metabolism. These monosaccharides are the main substrates for the production of various organic compounds including acetic acid, gluconic acid, some micronutrients, and glucuronic acid (Vijayaraghavan et al., 2000).

In many previous studies, the optimization of GlcUA production during the fermentative process was limited by the use of the whole traditional “mother-tea fungus” layer as the starter culture. This layer contains various types of microorganisms, and the particular yeasts or glucuronic acid producing bacterial strains had not been fully characterized (Vīna, Semjonovs, et al., 2013; Yavari et al., 2011). Also, kombucha may contain many contaminant microorganisms such as *Penicillium* spp. and *Candida albicans*, these contaminating strains can compete with the essential kombucha microorganism for nutrition and reduce the efficiency of

Abbreviations: AAB, acetic acid bacteria; GlcUA, glucuronic acid; KBC, kombucha; LAB, lactic acid bacteria; SBT, sweetened black tea.

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fermentation and glucuronic acid production. Moreover, *Penicillium* spp., a hyalohyphomycosis mold and *C. albicans* are infectious and opportunistic human pathogens (Kumamoto & Vinces, 2005; Schinabeck & Ghannoum, 2003). Home-cultured kombucha has a high risk of contamination due to non-aseptic operation and careless transfer among households. Although kombucha is good for health, its conventional culturing method, which uses the whole kombucha layer as the starter culture, make it difficult to control the unwanted microorganisms including pathogenic bacteria, yeasts and mold during fermentation (Dufresne & Farnworth, 2000; Eric & Jessica, 2013). To produce clean and safe GlcUA-rich kombucha, our first and foremost priority is to develop a simple and effective microbial symbiosis model. Since yeast and AAB play crucial roles in kombucha, it is important to isolate and select the best strains and characterize their fermentative features within the symbiosis before production on an industrial scale (Achi, 2005). These objectives were addressed in our study.

To manage the beneficial microbial strains in the production of GlcUA-rich kombucha, we isolated and screened for the most suitable yeast and bacterial strain and evaluated their ratios for GlcUA production. Another vital objective is to reduce the number of contaminant microorganisms in kombucha. Appropriate microbial control and biotechnology methods can manage the growth of microorganisms during the large-scale process and improve the quality of the traditional fermented tea. We believe that the results of this study will enable the beverage industry to produce higher quantities and qualities of healthy kombucha.

2. Materials and methods

2.1. Reagents, apparatus and medium preparations

Traditional kombucha with the whole cellulose membrane was kindly provided by biotechnology department, Faculty of Chemistry, University of Technology, Vietnam National University, and International University, Vietnam National University. Black tea (Lipton, Unilever, London) One liter of autoclaved sweetened black tea (SBT) contains 100 g of sucrose and extract of 1 g of Lipton black tea in boiling water. The new kombucha was cultured in the sweetened black tea medium by adding 5 g of the wet previous KBC layer to 100 mL of the tea volume. The eight-day fermented tea served as the source for microbial isolation.

2.2. Isolation, phenotypic characterization and identification of acetic acid bacteria

Pretreatment: 5 g of wet KBC layer was tore into small pieces, mixed with 5 mL solution of 10% (ml L^{-1}) cellulase enzymes (Sigma–Aldrich) then incubated for 2 h at 37 °C. The liquid sample was diluted then streaked onto MYP (D-mannitol 25 g L^{-1}, yeast extract 5 g L^{-1}, and bacteriological peptone 3 g L^{-1}, pH 5) agar plate and incubated for 5 days at 28–30 °C (Vuyst et al., 2008). Gram-negative bacterial strains which showed the positive results according to AAB's morphological, physiological and cultural characteristics were carried out in further biochemical tests.

H_2O_2 3% (ml L^{-1}) was used in catalase test. Single strain was spread on WL (Himedia, Mumbai, India) agar plates at pH 6.8 with the presence of 0.2 (g L^{-1}) bromolthymol blue (pH indicator) (Sigma–Aldrich) to determine acid production ability (Franke et al., 1999). The yellow zone-surrounding colonies indicate the low pH area. Water-soluble brown pigment determination in GYP broth culture (Kadere, Miyamoto, Oniang, Kutima, & Njoroge, 2008). The floating cellulose layers were stained with 2–3 drops of Schulze's reagent resulted in dark blue or black color (Amelio & Frank, 2002). Finally, the filtrates from 5-day fermented tea broth produced by the isolated strains were used for GlcUA detection. The selected AAB strains conferred the positive GlcUA production ability were selected to analyze in further experiments.

2.3. Isolation, selection and identification of yeast strain for symbiosis combination

Each 10 mL kombucha broth from top, middle, and bottom of fermented tea jar was collected to isolate yeast strains. The samples were diluted and then streaked onto 10 mg L^{-1} cycloheximide containing WL agar medium (Curtin, Bellon, Henschke, Godden, & Lopes, 2007). The incubation carried out in total 3–4 days at (29 ± 1) °C, the distinguished colonies were picked up based on the yeast morphology (Yarrow, 1998). Different isolated yeast strains were tested for the acid tolerant ability in pH-3 SBT medium, at (29 ± 1) °C for 24 h pH of the cultures was adjusted by the filtrate of 7-day fermented tea broth produced by the isolated AAB strains in previous section. Yeast's densities were determined by measuring OD value (600 nm) using UV-VIS Spectrophotometer (UV-2700, Shimazu, Japan). The highest density strain is the most highly adapted one which would be carried out in further experiments.

2.4. Screening for the optimal ratio between the two isolated strains for glucuronic acid production

Yeast and bacteria were cultured separately in a SBT medium and determined their densities by counting colonies on ML agar plates at every 6 hrs. The results were presented in form of log CFU. The inoculums of yeast and AAB were prepared separately in SBT medium for 72 hrs at 29 ± 1 °C. Then, their densities (number of living cell per milliliter) were determined as 23×10^8 (Yeast), 2×10^8 (AAB) (CFU mL^{-1}) before combining two strains for fermentation. The ratios of living cells between these two microbial strains were obtained by different transferring volumes from each inoculum into one final 50 mL-SBT medium containing glass. Different combinations of the two isolated strains were designed by increasing one's population while decreasing the other. The central point experiment was set at (5:5) ratio (Table 1).

All experimental samples were fermented at 29 ± 1 °C for 5 days before filtering through 0.22 μm spore size membrane and measuring GlcUA concentration by HPLC-MS. The unfermented tea and traditional kombucha served as the controls.

2.5. Study on the kinetic fermentation of the new designed symbiosis

The best ratio of microbial symbiosis was used to ferment SBT medium with 100 g L^{-1} sucrose at 30 °C, pH 4.5 and for 7 days to assess GlcUA, pH level, consumed sucrose, specific growth rate (μ), and velocity of glucuronic acid formation (ρ). GlcUA concentration

Table 1
The combination ratios between yeast and AAB living cells in reformation of kombucha symbiosis for glucuronic production.

	AAB	3	4	5	6	7
Yeast						
7		7Y:3A				
6			6Y:4A			
5				5Y:5A		
4					4Y:6A	
3						3Y:7A
0	A (no yeast)					

A: acetic acid bacteria, Y: yeast. Records of viable cells counted in the inoculums of yeast and AAB are 23×10^8 and 2×10^8 (CFU mL^{-1}), respectively. Different ratios reflect the different transferring volumes from the inoculum into the total 50-mL SBT medium containing glass for the symbiotic fermentation.