


Application of indigenous *Saccharomyces cerevisiae* to improve the black raspberry (*Rubus coreanus* Miquel) vinegar fermentation process and its microbiological and physicochemical analysis

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Abstract In order to improve the slow ethanol fermentation during acetic acid fermentation process of black raspberry vinegar (BRV), the microbiological and physicochemical aspects of the effects of indigenous *Saccharomyces cerevisiae* JBCC-21A were examined. The selected *S. cerevisiae* JBCC-21A showed better growth and ethanol production rates than the commercial yeast strains. The ethanol production rate was 3-times faster than the traditional method. Acetic acid fermentation by *S. cerevisiae* JBCC-21A began 10 days earlier than the traditional method and reached up to 60 g/L acetic acid. Bacterial counts revealed *Acetobacter pasteurianus* was the only dominant species throughout the inoculated acetic acid fermentation. The physicochemical and functional properties of the fermented vinegar using indigenous *S. cerevisiae* JBCC-21A maintained a high quality similar to the traditional method, while being the faster fermentation process. Thus, it is suggested that inoculation of the indigenous *S. cerevisiae* strain in order to shorten the fermentation time without affecting the quality of traditional BRV.

Keywords Black raspberry vinegar · Indigenous yeast · *Saccharomyces cerevisiae* · Ethanol fermentation · Acetic acid fermentation

Introduction

Technically, there are two types of methods used to produce vinegar: slow traditional processes and quick submerged (industrial purpose) methods. Unlike submerged methods, the vinegars produced by traditional static acetic acid fermentation are generally considered to be high quality due to diverse organic acids and amino acids being balanced during long-term surface culture fermentation (Lee et al., 2012). However, the traditional method, which is a lengthy process without microbial control delaying the alcoholic fermentation (AF) and acetic acid fermentation (AAF), may cause contamination and low acidity. Therefore, a technical strategy to achieve faster and higher acidity yield fermentation that maintains a high quality similar to the traditional method is needed.

Control of the ethanol production step by yeast inoculation is a priority in traditional vinegar fermentation, as it shortens the fermentation time and increases the safety of the product, thus ensuring the quality and reproducibility of the final product (Hidalgo et al., 2010). In yeast inoculated persimmon, balsamic, and strawberry vinegar fermentation, alcohol fermentation occurred faster in yeast-seeded vinegar due to the shorter lag phase without affecting the chemical composition or acetic acid bacteria (AAB) diversity (Hidalgo et al., 2010; 2012).

Many reports have established that the use of indigenous *S. cerevisiae* strains is preferred by many winemakers since active commercial dried yeasts reduce both the biodiversity of the strains and the wine complexity (Frezier and Dubourdieu, 1992). They also possess high fermentation capability, as native yeast populations are strongly adapted to specific environments, thus having important implications for the use of autochthonous wild yeast strains as starters (Nadal et al., 1996). The use of starter cultures has

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improved the reproducibility and predictability wine quality (Di Maro et al., 2007).

Traditional Muju black raspberry vinegar (TMBV) in Korea has been manufactured empirically with black raspberry extract (BRE) to facilitate the growth of desirable microorganisms in a crock using the back-slopping method with seed vinegar. Due to the health-related effects of black raspberry and black raspberry vinegar (BRV) containing nutritious components and various physiological activities, BRV has received great attention for its anti-oxidative, anti-tumor activity, anti-inflammatory, anti-high lipid, anti-high sugar, and antimicrobial activity (Hong et al., 2012; Lim et al., 2012). However, slow spontaneous alcohol fermentation affected slow starting acetification, which resulted in a low final total acidity even with a long fermentation time. To establish an effective BRV fermentation process, a reduction in the length of the AF and the control of the process are needed (Hidalgo et al., 2012).

In this study, we isolated the indigenous starter yeast from traditionally prepared TMBV and examined their biochemical and fermentation properties to improve the BRV fermentation process. Moreover, we also applied the selected indigenous starter yeast to the pilot-scale BRV fermentation process.

Materials and methods

Yeast strains

The yeast strains were isolated from traditionally prepared black raspberry wine and TMBV by using YM agar plates (Difco Co., Detroit, MI, USA) containing 25 U/mL penicillin–streptomycin (Sigma) to inhibit bacterial growth were used. After incubating for 2–3 days at 29 °C, yeast-like colonies were randomly picked and cultivated in YM broth for 48 h. Isolates were kept at – 80 °C in a deep freezer. *S. cerevisiae* KACC30008 and commercial dried yeast *S. cerevisiae* no. 7013 (DSM Food Specialties, INRA NARBONNE, Servian, France) were used as the standard control yeast strains.

Glucose, metabisulfite and ethanol tolerance

To determine the environmental tolerance capability of the strains, the yeast cells were grown in YM broth with different concentrations of glucose (200 and 300 g/L), metabisulfite (200 and 500 mg/L), and ethanol (0, 5, 10, and 15%, v/v). Yeast strains were cultivated at 29 °C, 180 r/min for 2 days. The absorbance was monitored at 12, 24 and 48 h at 600 nm using a spectrophotometer (UV–VIS Spectrophotometer, Beckman Coulter, USA).

Biogenic amine (BA) analysis

The BA standards, including tryptamine, 2-phenylethylamine, putrescine, cadaverine, histamine, serotonin, tryptamine, spermidine, spermine, L-noradrenaline, and dopamine, were obtained from Sigma-Aldrich (St. Louis, MO, USA). An analysis of the concentration of BA was carried out using the dansyl chloride derivatization reaction. After derivatization of the samples, an aliquot of samples was submitted to high-pressure liquid chromatography (Agilent Technologies, Santa Clara, CA, USA) with a UV detector and CapcellPak C18 Column (4.6 × 250 mm, 5 µm, Shiseido Co., Tokyo, Japan), as described previously (Moon et al., 2010).

Ethanol analysis

Ethanol production by yeast strains was analyzed using gas chromatography (HP 6890, Hewlett Packard, CA, USA) with a flame ionization detector (FID) and a DB-5 capillary column (30 m × 0.25 mm, 0.25 µm; J&W Scientific, CA, USA), following the methods of Song et al. (2016b). The temperature was programmed to rise from 70 to 150 °C at a rate of 20 °C/min, followed by an increase to 220 °C, which was held for 1 min.

Yeast identification

Restriction fragment length polymorphism (RFLP), internal transcribed spacer (ITS), and 26S rDNA sequencing of yeasts were performed as described previously (Solieri et al., 2006; Song et al., 2016b).

Black raspberry wine and vinegar fermentation process

The BRE and seed vinegar obtained as described previously (Song et al., 2016a; 2016b). The BRE was heated at 85 °C for 15–20 min, and then used for black raspberry wine (BRW) fermentation. Wine fermentation was performed using a two-step fermentation process. First, the log 8–9 CFU/mL of starter yeast inoculated in 20°Brix of BRE and incubated it at 25 °C, 160 rpm for 48 h. Then, the AF was performed in the stationary phase for 27 days. The obtained BRW (700 L) was mixed with 100 mL/L of seed vinegar. AAF was performed in a bioreactor crock at 23 °C without agitation. Every week, 50 mL samples were collected and immediately used for chemical analysis, traditional microbial counts and culture-independent microbiological analysis. The total soluble solids (°Brix), pH and acidity were analyzed as previously described by Song et al. (2016b).

Yeast and acetic acid bacteria counts

Samples obtained from AAF were serially diluted with sterilized water and spread onto agar plates to isolate microorganisms. YM agar plates containing 25 U/mL penicillin–streptomycin (Sigma) as described above were used for yeast counting. For AAB isolation, two different media, GYC agar (5% glucose, 1% yeast extract, 3% ethanol, 0.8% agar) and GYEC agar plates (5% glucose, 1% yeast extract, 3% ethanol, 1.5% calcium carbonate, 0.8% agar), were used for bacterial counting. Counting was conducted after incubating for 2–3 days at 29 °C.

General and functional component analysis

Mineral contents, free sugars, and organic acids were analyzed following the methods of Song et al., (2016b). The total polyphenol contents of the samples were determined using the Folin–Ciocalteu method with gallic acid as a standard phenolic compound (Folin and Denis, 1912). The total flavonoid content of the BRV samples was determined using a colorimetric method described in the literature (Davis, 1947) and catechin used as a standard. The DPPH radical scavenging activity was measured as described by Meda et al. (2005).

DNA extraction and PCR-DGGE analysis

Total DNA for DGGE analysis was extracted directly from the vinegar samples using the i-genomic soil DNA extraction mini kit (Intron biotechnology, INC., Seoul, Korea) following the manufacturer's instructions. DNA from each single band was extracted and purified using the Genomic DNA Purification kit from Promega (Madison, WI, USA). For yeast identification, DNA extracted from the DGGE gels was amplified by PCR with NL1/NL4 and NL1/LS2 primers, respectively, using the previously reported protocol (Mills et al., 2002). The DNA sequences of bacteria were amplified with the primer pair 357f-GC clamp and 517r following a reported protocol (Muyzer et al., 1993). The DGGE analysis of PCR amplicons was carried out following the method of De Vero et al. (2006).

Statistics

The data were analyzed using the Student's *t* test with the SPSS version 20.0 software. The results of the analyses, which were performed in triplicate, were expressed as the mean \pm standard deviation (SD). The differences among groups were assessed using Duncan's multiple range tests.

Results and discussion

Selection, characterization and identification of the indigenous strain

To select starter yeast for rapid and controlled AF, approximately 200 yeast strains were isolated from traditionally fermented black raspberry wine or TMBV in this study. The JBCC-21A strain was finally selected due to its high growth properties with glucose or metabisulfites, ethanol productivity (13%, v/v), and BA degradation activity (Table 1). In particular, the selected JBCC-21A strain was able to remove 100% cadaverine, spermidine, and 15% histamine, which was 3–8 times lower than the normal toxic dose (Soufleros et al., 1998). The strain was identified via ITS-RFLP, ITS, and 26S rDNA sequencing, showing 99% homology with *S. cerevisiae*.

Ethanol fermentation properties of the selected indigenous *S. cerevisiae* JBCC-21A strain on black raspberry extract

During AF, *S. cerevisiae* JBCC-21A showed similar growth properties than *S. cerevisiae* KACC30008 (Fig. 1C), which initially showed identical growth. However, *S. cerevisiae* KACC30008 eventually began decreasing dramatically starting from day 14 and *S. cerevisiae* no. 7013 showed slow growth. *S. cerevisiae* JBCC-21A and *S. cerevisiae* no. 7013 showed similar sugar consumption, but that of *S. cerevisiae* KACC30008 was significantly low. These results clearly show that indigenous *S. cerevisiae* JBCC-21A has better adaptation and fermentation capacity in the black raspberry environment than do standard control yeast strains.

As shown in Fig. 1B, the ethanol concentration increased up to 12.2% (v/v) when *S. cerevisiae* JBCC-21A was used as a starter until day 15, also exhibiting a decrease in total sugar (Fig. 1A). Commercial yeast *S. cerevisiae* no. 7013, which is broadly used in industrial fermentation, only produced 8% (v/v) ethanol in BRE (Fig. 1B); this value is twice as low as the ethanol production rate for the selected *S. cerevisiae* JBCC-21A. The BRW prepared using *S. cerevisiae* JBCC-21A (BRW-21A) showed a slightly lower pH than that of *S. cerevisiae* KACC30008 (BRW-K30008) and *S. cerevisiae* no. 7013 (BRW-7013) (Fig. 1D). As shown in Fig. 1D, the total acidity of BRW-21A increased up to 6.0 g/L on day 18, which was almost identical to that for grape wine at 5.6–9.6 g/L (Kim et al., 2010). BRW-K30008 showed a slightly lower total acidity (4.4 g/L), whereas BRW-7013 increased up to 8.5 g/L through day 23. Hence, the isolated indigenous strain *S. cerevisiae* JBCC-21A was

Table 1 Characterization of indigenous *Saccharomyces cerevisiae* JBCC-21A and standard control yeasts

Tolerance	JBCC-21A	<i>S. cerevisiae</i> no. 7013	<i>S. cerevisiae</i> KACC30008
Ethanol production	Over 10% (v/v)	15.8–16.4 (% v/v) (Lee and Ahn, 2009) 11 ± 0.1 (% v/v) from 21°Brix of the must) (Lee et al., 2006)	16 (% v/v) (Jang et al., 2011)
Glucose tolerance (g/L)		–	–
200	+, OD ₆₀₀ < 15		
300–500	++, 20 < OD ₆₀₀		
Na ₂ S ₂ O ₅ tolerance (mg/L)			–
200	++, 7 < OD ₆₀₀	≤ 4 mM SO ₂ (active growth) (Barbosa et al., 2014)	
500	++, 7 < OD ₆₀₀		
Ethanol tolerance (% v/v)			–
5	+++ , 6 < OD ₆₀₀	≤ 10 (% v/v) (medium growth)	
10	+, 1 < OD ₆₀₀		
15	–, OD ₆₀₀ < 1	> 12.5 (% v/v) (no growth) (Barbosa et al., 2014)	
Biogenic amine degradation		–	–
Cadaverine	100%		
Histamine	15.07%		
Spermidine	100%		
Identification			
ITS-RFLP (5.8S rDNA)	<i>S. cerevisiae</i> (100%, JN083825)		
ITS (ITS1, ITS4)	<i>S. cerevisiae</i> 99%		
26S rDNA	<i>S. cerevisiae</i> 100%		

demonstrated to have efficient ethanol fermentation properties upon BRE.

Physicochemical changes by *S. cerevisiae* JBCC-21A during BRV fermentation

The effects of *S. cerevisiae* JBCC-21A on physicochemical changes during BRV fermentation at a pilot scale (700 L, stainless barrel) are shown in Table 2. After inoculating activated *S. cerevisiae* JBCC-21A, the ethanol concentration increased up to 12.6% (v/v) until day 27, which was similar to that of the traditional fermentation method (Song et al., 2016b). However, the ethanol concentration by the traditional method reached its maximum concentration very slowly until day 59, which was almost three times longer than *S. cerevisiae* JBCC-21A. The levels of soluble solids (°Brix) decreased up to 9.5°Brix and reached a maximum of 12.6% (v/v) ethanol until the final stage of AF (day 27). After the ethanol production step was almost finished at 35 days, the seed vinegar (18.8°Brix) was inoculated to initiate the second step of AAF. Acetic acid production was activated after the acidity reached 2%,

which is the appropriate initial acidity for acetic acid fermentation (Hong et al., 2012), and the total acidity increased from 20 g/L (day 35) to 60 g/L (day 144). Consequently, these results suggest that a larger acidity can be achieved quicker and more efficiently via the BRV fermentation process by adopting the isolated *S. cerevisiae* JBCC-21A compared to TMBV (Song et al., 2016b).

Analysis of BRV fermented using indigenous *S. cerevisiae* JBCC-21A

The biochemical and functional properties of BRV fermented using indigenous *S. cerevisiae* JBCC-21A were analyzed to understand the effects of indigenous *S. cerevisiae* JBCC-21A on the BRV fermentation process. The remaining carbon source of BRV was fructose (2.07 g/L), whereas glucose was not detected (Table 3). The total acidity and final ethanol concentration of TMBV were 44 g/L and 1.9% (v/v), respectively (Song et al., 2016b), while those for BRV were 60 g/L and 0.93% (v/v), respectively. As shown in Table 3, acetic acid (2.82 g/L) and oxalic acid (3.38 g/L) were the two major organic

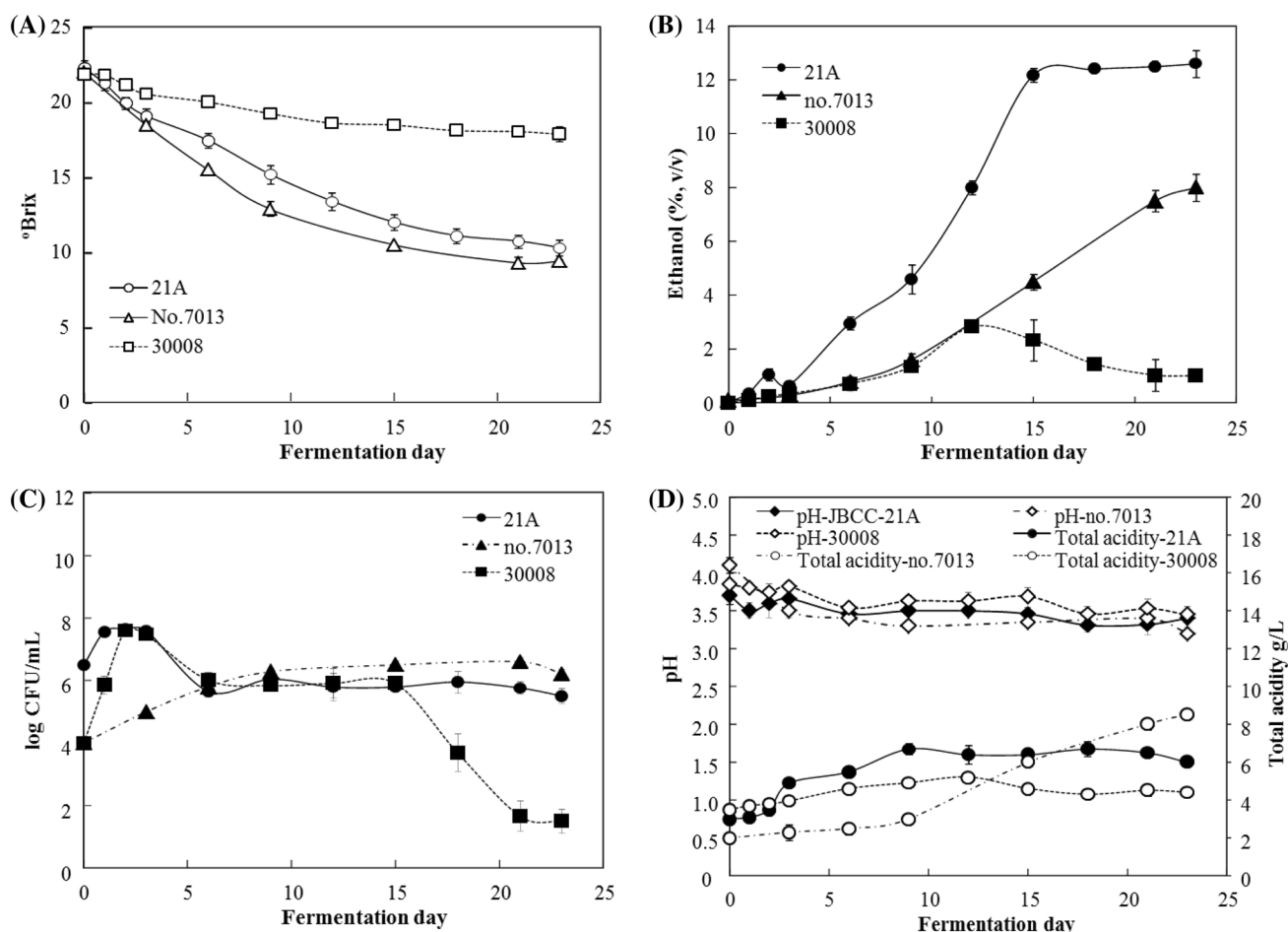


Fig. 1 Physicochemical changes in sugar consumption (A), ethanol production (B), yeast population (C), total acidity and pH (D) during alcohol fermentation by indigenous strain and standard yeasts. 21A,

Saccharomyces cerevisiae JBCC-21A; no. 7013, commercial *S. cerevisiae* no. 7013; 30008, type strain *S. cerevisiae* KACC30008

acids in BRV. Acetic acid (59.11 g/L), oxalic acid (7.7 g/L) and citric acid (1.18 g/L) were mainly generated after AAF, but both malic acid and lactic acid were reduced. The different organic acid profiles obtained in this study, which were comparable to those for the traditional method (Song et al., 2016b), could be explained by the shortened AF period and ethanol oxidation. In particular, the faster oxidation of some organic acids by AABs may have been due to the shortened AAB proliferation and ethanol consumption period caused by large and fast ethanol production (Giudici et al., 2009). Similarly, different organic acid and volatile compounds between traditional balsamic vinegar (TBV) and commercial balsamic vinegar (BV), which were manufactured using different methods were also observed (Li et al., 2015). The large amount of acetic acid may be due to the rapid consumption of ethanol obtained from the inoculated AF. Further, some of the changes in the organic acids are a result of the acetate overoxidation during diauxic growth of the *Acetobacter* species (Li et al., 2015).

The minerals, Mg reacted with citric acid presented in BRV may stabilize the vinegar forming soluble complexes (Casale et al., 2006) and higher Fe concentration plays a fundamental role as redox catalysts on the antioxidant properties of BRV.

As shown in Fig. 2, the BRV fermented using indigenous *S. cerevisiae* JBCC-21A contained significantly higher phenolic content than BRW ($p < 0.001$), and the total flavonoid contents of BRW and BRV were 1.68 and 2.56 g/L, respectively ($p < 0.01$). The total polyphenol content was higher than 25.19 mg/100 mL for the black raspberry vinegar from the Gochang area (Hong et al., 2012). The total polyphenol, total flavonoid, and DPPH radical scavenging activities of TMBV were 1.98 g/L, 1.91 g/L, and 1.63 g AEAC/L (g of ascorbic acid equivalent antioxidant content, data not shown), respectively (Song et al., 2016b); however, in this study, BRV fermented by indigenous *S. cerevisiae* JBCC-21A showed higher values of 2.61 g/L, 2.56 g/L, and 1.88 g AEAC/L, respectively. This result was in agreement with the higher

Table 2 Changes of microbial enumeration and physicochemical components during black raspberry vinegar fermentation process using *Saccharomyces cerevisiae* JBCC-21A

Fermentation method	Stage	Sampling Day	Day	Yeast (log CFU/mL)	Ethanol (%)	Soluble solids (°Brix)	AAB (log CFU/mL)	Total titratable acidity (g/L)	pH
Inoculated alcohol fermentation	Initial	0	0	5.48 ± 0.32	0.1 ± 0.00	22.3 ± 0.01	< log 3.0	2.0 ± 0.5	4.1 ± 0.02
		1	10	7.59 ± 0.21	4.6 ± 0.12	15.2 ± 0.02	< log 3.0	3.0 ± 0.1	3.3 ± 0.01
	Middle	2	20	5.78 ± 0.30	12.5 ± 0.23	12 ± 0.01	< log 3.0	8.2 ± 0.2	3.36 ± 0.01
		3	27	5.5 ± 0.22	12.6 ± 1.21	10.3 ± 0.02	< log 3.0	13.1 ± 2.0	3.2 ± 0.03
Seed vinegar inoculated acetic acid fermentation	Initial	4	35	5.47 ± 0.26	7.16 ± 1.30	10.3 ± 0.02	3.5 ± 0.22	19.5 ± 2.1	2.97 ± 0.01
		5	45	5.38 ± 0.25	7.17 ± 2.10	10.6 ± 0.03	3.5 ± 0.32	24.3 ± 2.2	3.13 ± 0.01
		6	52	5.47 ± 0.21	7.44 ± 1.54	11.3 ± 0.02	4.5 ± 0.05	25.8 ± 3.3	3.09 ± 0.02
		7	59	5.65 ± 0.31	7.0 ± 2.41	10.9 ± 0.04	4.85 ± 0.21	27.4 ± 3.5	3.06 ± 0.03
	Middle	8	72	5.15 ± 0.33	6.0 ± 2.30	10.9 ± 0.03	5.01 ± 0.30	30.4 ± 2.4	3.05 ± 0.02
		9	77	4.88 ± 0.26	4.79 ± 1.10	10.8 ± 0.05	5.2 ± 0.22	30.4 ± 5.6	3.05 ± 0.00
		10	87	4.21 ± 0.24	3.62 ± 1.01	10.2 ± 0.01	5.1 ± 0.25	27.4 ± 3.1	3.02 ± 0.01
		11	95	3.8 ± 0.28	3.5 ± 2.21	10.0 ± 0.02	5.72 ± 0.34	31.2 ± 1.2	2.96 ± 0.00
		12	104	3.52 ± 0.35	3.15 ± 1.41	10.0 ± 0.03	5.48 ± 0.24	35.0 ± 2.1	3.04 ± 0.01
		13	119	2.83 ± 0.24	2.8 ± 0.23	10.1 ± 0.02	5.71 ± 0.22	41.0 ± 0.8	2.96 ± 0.00
	Final	14	144	2.71 ± 0.22	0.93 ± 0.02	9.5 ± 0.03	5.8 ± 0.30	62.3 ± 1.2	2.94 ± 0.02
		0	0	5.0 ± 0.05	0.2 ± 0.05	19.5 ± 0.01	0.5 ± 0.33	0.4 ± 0.05	4.0 ± 0.05
	Traditional alcohol fermentation ^a	1	10	7.29 ± 0.2	4.5 ± 0.40	14.7 ± 0.03	1.5 ± 0.41	1.06 ± 0.1	3.16 ± 0.1
		2	20	7.37 ± 0.3	4.93 ± 0.06	7.8 ± 0.04	1.86 ± 0.22	1.12 ± 0.2	3.26 ± 0.2
3		27	7.29 ± 0.1	5.25 ± 0.02	6.0 ± 0.03	2.0 ± 0.12	1.37 ± 0.2	3.22 ± 0.2	
Traditional acetic acid fermentation ^a	4	35	7.58 ± 0.3	11.81 ± 0.42	5.9 ± 0.04	2.65 ± 0.34	1.55 ± 0.1	3.13 ± 0.05	
	5	45	6.79 ± 0.05	11.56 ± 0.31	5.6 ± 0.02	3.0 ± 0.50	1.98 ± 0.3	3.21 ± 0.05	
	6	52	6.63 ± 0.2	11.71 ± 0.33	5.9 ± 0.03	3.34 ± 0.31	1.98 ± 0.2	3.16 ± 0.1	
	7	59	6.82 ± 0.4	12.89 ± 0.52	5.7 ± 0.01	3.50 ± 0.24	2.13 ± 0.05	3.09 ± 0.2	
	8	72	3.65 ± 0.05	6.14 ± 0.13	5.7 ± 0.03	3.78 ± 0.12	2.28 ± 0.1	3.09 ± 0.1	
	9	77	3.5 ± 0.05	5.82 ± 0.00	5.7 ± 0.02	4.85 ± 0.32	2.43 ± 0.1	3.09 ± 0.05	
	10	87	3.5 ± 0.2	5.49 ± 0.05	5.4 ± 0.05	5.51 ± 0.21	2.13 ± 0.2	3.04 ± 0.2	
	11	95	3.3 ± 0.3	5.16 ± 0.21	5.1 ± 0.03	5.54 ± 0.16	2.66 ± 0.3	2.96 ± 0.2	
	12	104	3.0 ± 0.3	3.72 ± 0.21	5.1 ± 0.03	5.82 ± 0.23	2.89 ± 0.05	3.05 ± 0.1	
	13	119	2.6 ± 0.1	3.50 ± 0.10	5.3 ± 0.01	5.62 ± 0.22	3.19 ± 0.1	3.0 ± 0.05	
	14	144	2.3 ± 0.4	1.97 ± 0.11	5.4 ± 0.04	5.16 ± 0.30	4.41 ± 0.2	3.02 ± 0.1	

^aTraditional fermentation method (Song et al., 2016b)

total polyphenol values caused from inoculated wines compared to those of vinegars from spontaneous fermentation (Ubeda et al., 2011). This result may be due to the microbial changes accompanying the physicochemical components. Some of the non-*Saccharomyces* strains that resulted from environmental changes after adding seed vinegar also affected the values of polyphenol, anthocyanin and antioxidant activity (Li et al., 2015; Tu et al., 2005).

Microbiological characterization during BRV fermentation by *S. cerevisiae* JBCC-21A

After inoculating *S. cerevisiae* JBCC-21A, their population rapidly proliferated until 10 days, and decreased to log 2.71 CFU/mL in the final BRV as shown in Table 2. Vinegar fermentation followed with rapid growth of AAB population to log 5.8 CFU/mL. DGGE analysis showed the 14 bands, which identified each of the seven yeasts and seven bacterial species (Fig. 3). As expected, *S. cerevisiae* JBCC-21A inoculated at an early stage of AF dominated until day 27 (Fig. 3A), indicating that *S. cerevisiae* JBCC-21A adapted to BRV fermentation successfully. However,

Table 3 Biochemical analysis of black raspberry wine and vinegar using inoculated method

Samples	Residual sugars (g/L)			Organic acid (g/L)					Mineral (ppb)	
	Glucose	Fructose	Sucrose	Acetic acid	Oxalic acid	Citric acid	Lactic acid	Malic acid	Mg	Fe
BRW	nd	2.49 ± 0.6 ^a	nd	2.82 ± 1.36 ^b	3.38 ± 1.77 ^a	0.06 ± 0.01 ^b	0.77 ± 0.08	0.5 ± 0.1	33,308 ^b	2511 ^b
BRV	nd	2.07 ± 0.0 ^a	nd	59.11 ± 8.48 ^a	7.7 ± 4.15 ^a	1.18 ± 0.29 ^a	nd	nd	83,270 ^a	4813 ^a
TMBV ^c	nd	10.01 ± 1.04	nd	72.12 ± 19.58	nd	nd	1.81 ± 1.38	1.07 ± 0.03		

Data is expressed as mean ± standard deviation (n = 3)

BRW black raspberry wine fermented by indigenous yeast, *S. cerevisiae* JBCC-21A, BRV black raspberry vinegar fermented by 100 mL/L seed vinegar inoculation into BRW

^{a,b}Small letters in the same column indicate significant differences of values between two samples by student's t-test ($p < 0.05$)

^cTMBV, traditional Muju black raspberry vinegar (Song et al. 2016b)

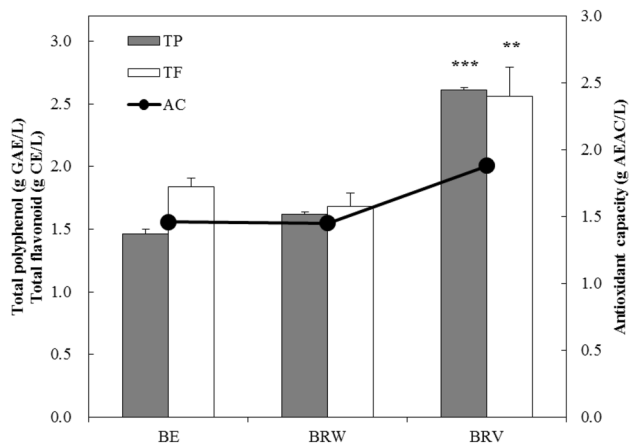
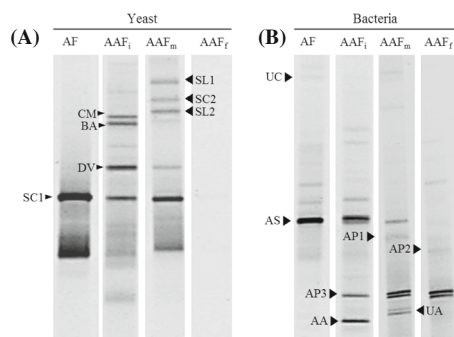


Fig. 2 Functional component analysis of black raspberry wine and vinegar fermented by inoculated method. BRE, black raspberry extract; BRW, black raspberry wine fermented by indigenous yeast, *Saccharomyces cerevisiae* JBCC-21A; BRV, black raspberry vinegar fermented by 100 mL/L seed vinegar inoculation into BRW; TP, total polyphenol expressed by gallic acid equivalent (GAE); TF, total flavonoid expressed by catechin equivalent (CE); AC, ascorbic acid equivalent antioxidant content (AEAC) with DPPH radical scavenging activity. *, small asterisk in the same bar indicate significant differences of values between two samples by student's t-test (** $p < 0.01$; *** $p < 0.001$)

other yeasts, including *Candida magnolia* (CM), *Botrytis aclada* (BA), *Denhamia viridissima* (DV), and *Saccharomycodes ludwigii* (SL1, 2), also appeared in the initial (AAF_i) and middle (AAF_m) stages of static fermentation. However, those yeasts gradually disappeared without affecting the dominant species of *S. cerevisiae* JBCC-21A during the vinegar fermentation period. Previous studies have shown that non-*Saccharomyces* yeast species such as the genera *Candida* and *Saccharomycodes* appeared during the initial and middle stages of acetification for white wine vinegar, balsamic vinegar, wine vinegar and kombucha vinegar, often showing more beneficial effects with positive metabolic activities (Li et al., 2015). During AF (Fig. 3B), several ethanol-tolerant *Acetobacter* species

such as *Acetobacter nitrogenifigens* (NR043086.1), *A. indonesiensis* (NR028626.1), *A. tropicalis* (NR036881.1), *A. orleanensis* (NR028614.1), *A. malorum* (NR025513.1), *A. cerevisiae* (NR025512.1), and *A. estunensis* (NR042113.1) could be detected due to the high ethanol concentration. After adding seed vinegar, *A. aceti*, which was the most dominant species in black raspberry seed vinegar (Song et al., 2016a), quickly disappeared, allowing *A. pasteurianus* to be the dominant bacterial species throughout the end stage of vinegar fermentation. *A. pasteurianus* was more successfully grown compared to *A. aceti*, which may be due to their different acetic acid tolerances. The increased stressful acidity and pH during AAF resulted in a more suitable environment for AAB, and yeasts were not detected during the final stage of the acetification process (AAF_f). Interestingly, in our previous study (Song et al., 2016b), *Acetobacter* sp. was abundant in the initial and middle stages and *Komagataeibacter xylinus* was observed in the final stage of TMBV fermentation (Song et al., 2016b), whereas inoculation of indigenous *S. cerevisiae* JBCC-21A contributed to AAB succession. Although commercial wine yeast inoculation did not influence the AAB diversity or chemical composition of the vinegars in a previous study by Hidalgo et al. (2012), yeast inoculation during AF in our study may have affected AAB proliferation due to the rapidly-occurring and larger ethanol concentration (over 12%). *Gluconacetobacter* sp. can be easily dominated under harsh conditions such as high alcohol or high acidity conditions (Hidalgo et al., 2012), whereas *A. pasteurianus* also can grow quickly in blueberry wine vinegar (Hidalgo et al., 2013a), traditional wine vinegars (Vegas et al., 2010), traditional balsamic vinegar (Gullo et al., 2009), and Chilean wine vinegar (Ilabaca et al., 2008) due to their strong oxidizing activity for ethanol into acetic acid compared to glucose. Furthermore, *A. pasteurianus* was not only detected in vinegars with low concentrations of acetic acid (3–4%) (Haruta et al., 2006), but was also found as indigenous organisms in



Groups	Fermentation method	Process			
		AF	AAFi	AAFm	AAFr
(A) Yeast	Inoculated	<i>S. cerevisiae</i> (SC1)	<i>S. cerevisiae</i> (SC1) <i>C. magnoliae</i> (CM) <i>B. aclada</i> (BA) <i>D. viridissima</i> (DV)	<i>S. cerevisiae</i> (SC2) <i>S. ludwigii</i> (SL1) <i>S. ludwigii</i> (SL2)	-
	Traditional ^a	<i>S. cerevisiae</i> (SC3)	<i>S. cerevisiae</i> (SC3)	<i>S. cerevisiae</i> (SC3)	-
(B) Bacteria	Inoculated	<i>Acetobacter</i> sp. (AS) Uncultured bacteria (UC)	<i>A. pasteurianus</i> (AP) <i>A. aceti</i> (AA) <i>Acetobacter</i> sp. (AS)	<i>A. pasteurianus</i> (AP) <i>Acetobacter</i> sp. (AS) Uncultured <i>Acetobacter</i> sp. (UA)	<i>A. pasteurianus</i> (AP)
	Traditional ^a	<i>A. pasteurianus</i> (AP4)	<i>A. pasteurianus</i> (AP5)	<i>A. pasteurianus</i> (AP5)	<i>K. xylinus</i> (KX)

^a Traditional fermentation method (Song et al., 2016b)

Fig. 3 Yeast (A) and acetic acid bacteria (B) species monitoring by PCR/DGGE analysis during black raspberry vinegar fermentation using *Saccharomyces cerevisiae* JBCC-21A. AF, alcohol fermentation; AAF_i, initial stage of acetic acid fermentation; AAF_m, middle stage of acetic acid fermentation; AAF_r, final stage of acetic acid fermentation. The closest relatives of the fragments sequenced (% identical nucleotides compared to sequences retrieved from the GenBank database) are described in separated table: (Top) (A) SC1, *S. cerevisiae* (99%, JX068683.1); CM, *C. magnolia* (97%, AJ749827.1); BA, *B. aclada* (99%, KC311471.1); DV, *D. viridissima*

(97%, EU328648.1); SC2, *S. cerevisiae* (97%, JX068683.1); SL1, *S. ludwigii* (95%, JN248612.1); SL2, *S. ludwigii* (98%, JN248612.1); (B) AS, *Acetobacter* sp. (96%); UC, uncultured bacteria (100%, KC872586); AP1, *A. pasteurianus* (98%, JQ513826.1); AA, *A. aceti* (96%, AJ012542.1); AP2, *A. pasteurianus* (98%, JQ513826.1); UA, uncultured *Acetobacter* sp. (97%, JQ253455.1); AP3, *A. pasteurianus* (98%, JQ513826.1). (Bottom) SC3, *S. cerevisiae* (99%, JX068683.1); AP4, *A. pasteurianus* (99%, JQ513826.1); AP5, *A. pasteurianus* (99%, JQ513826.1); KX, *Komagataeibacter xylinus* (100%, NR_074338.1)

wine and fruit vinegars containing 5.5–6% acidity (Hidalgo et al., 2013b; Li et al., 2015; Vegas et al., 2010). Thus, the different AAB patterns (compared to the traditional method) observed in this study depend on the inoculated fermentation method, with *A. pasteurianus* continuously being the dominant species due to the higher alcohol and higher acidity conditions during BRV fermentation.

In this study, we suggested that indigenous starter yeast *S. cerevisiae* JBCC-21A with the seed vinegar of black raspberry was more effective for fast AF than the traditional black raspberry vinegar fermentation process. Our results indicated that the inoculated BRV fermentation process with the selected indigenous strain is an efficient approach for the fast AF step, therefore enhancing the overall BRV fermentation process without affecting the quality of traditional BRV. The native starter yeast, *S. cerevisiae* JBCC-21A inoculated with AF, showed higher viability and ethanol production than commercial yeast. Although the fungal and bacterial diversity were changed, *S. cerevisiae* JBCC-21A dominated while the *A. pasteurianus* species completed the acetification of BRV

fermentation. Microbial succession was simpler compared to the traditional method, while showing similar or higher functional compounds and antioxidant activity. These results demonstrate the potential for industrial production of a stabilized and fast BRV fermentation process using indigenous yeast as a starter culture with seed vinegar.

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