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ORIGINAL RESEARCH PAPER

Co-fermentation of grape must by *Issatchenkia orientalis* and *Saccharomyces cerevisiae* reduces the malic acid content in wine

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Abstract Grape must was fermented by a mixed culture of *Saccharomyces cerevisiae* W-3 (a wine yeast) and *Issatchenkia orientalis* KMBL 5774 (a malic acid-degrading yeast). Co-fermentation with 1:1 (v/v) inoculum ratio of W-3 and KMBL 5774 decreased malic acid to 0.33 mg/ml from 1.1 mg ml with W-3 alone. Ethanol production was the same in both cases (7.8%, v/v). Acetaldehyde, 1-propanol, 2-butanol and isoamyl alcohol all decreased, with an increase in methanol, in the co-fermented wine. Sensory evaluation showed a higher score in the wine fermented with 1:1 (v/v) inoculum ratio than those obtained by 4:1 (v/v) inoculum ratio or W-3 alone.

Keywords Co-fermentation · Grape · *Issatchenkia orientalis* · Malic acid degradation · Wine

Introduction

Malic acid, which is the major organic acid together with tartaric acid in the grape, is sometimes detrimental to the quality of wines when present at high concentrations in some varieties (Pretorius 2000; Ruffner 1982). Several grape varieties contain considerable amounts of malic acid and grapes grown in the cooler regions contain higher amounts of malic acid than those grown in the warmer regions (Ruffner 1982). Excessive amounts of malic acid (15–16 mg/ml) were detected in grapes grown during exceptionally cold summer in cool regions (Gallander 1977). Campbell's Early grape, the major grape variety in Korea, contains a large amount of malic acid to negatively affect the wine quality (Kim et al. 1999).

Saccharomyces sp., the major species used for the wine brewing, cannot degrade malic acid efficiently. Typically, strains of *Saccharomyces* sp. are regarded as inefficient metabolisers of extracellular malic acid (Delcourt et al. 1995; Volschenk et al. 1997; 2001). Moreover, some strains of *Saccharomyces* sp. synthesize malic acid (Fatichenti et al. 1984; Schwartz and Radler 1988). Therefore, its level in wine grapes is of concern to wine makers and researchers to improve the sensory quality of wines (Thornton and Rodriguez 1996). There have been a large number of researches on the isolation and characterization of microorganisms, especially yeast strains, degrading malic acid. Among the yeasts degrading malic acid in the presence of glucose, are *Schizosaccharomyces pombe*, *Schizosaccharomyces malidevorans* and *Zygosaccharomyces bailii* (Baranowski and Radler 1984; Taillandier et al. 1988; Thorton and Rodriguez 1996). Although *Schizosaccharomyces* sp. has been intensively studied for reduction of malic acid

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content in wine, none of the studies have been successful because of its off-flavor production (Gallander 1977; Radler 1993).

Previously, *Issatchenkia orientalis* KMBL 5774 degrading malic acid was isolated from Korean grape wine pomace. It could degrade malic acid rapidly in the media containing malic acid as the sole carbon and energy source (Seo et al. 2007). In this study, fermentations of Korean grape must by *I. orientalis* KMBL 5774 as well as mixed cultures of *I. orientalis* KMBL 5774 and *S. cerevisiae* W-3 (an industrial wine yeast) was investigated to test a possible application of the strain to the fermentation of grapes containing high malic acid content.

Materials and methods

Strains and growth conditions

Issatchenkia orientalis KMBL5774, used as a malic acid-degrading yeast, was described in detail previously (Seo et al. 2007). An industrial wine yeast strain, *Saccharomyces cerevisiae* W-3, was used for the co-fermentation of Korean grape must was from the laboratory stock culture. The yeasts were grown at 30°C in YPD liquid media consisted of 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone and 2% (w/v) glucose. For the preparation of yeast inocula for the wine fermentation, yeast cells were grown at 30°C for 2 days in 3% (w/v) malt extract Difco broth with shaking at 150 rpm.

Wine fermentation

For the wine fermentation, Campbell's Early grapes grown in Korea were used. Potassium metabisulfite was directly added at 200 μg/ml to grape must (10 kg) obtained by de-stemming and crushing of grapes. Yeast cells, grown at 30°C for 2 days in malt extract media, were harvested by centrifugation and used to inoculate the grape must at 5% (v/v). For the wine fermentation by the mixed culture of *S. cerevisiae* W-3 and *I. orientalis* KMBL5774, the yeast cells were inoculated as 4:1, 1:1 and 1:4 ratio based on the culture volume of the two strains. After the inoculation, alcohol fermentation was carried out at 20°C for 15 days.

Determination of viable counts of *S. cerevisiae* and *I. orientalis* during co-fermentation

Viable counts of *S. cerevisiae* and *I. orientalis* were directly determined based on their different colony color and morphology on YPD plates using 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining by the method of Bochner and Savageau (1976). Diluted wine samples were spread on the YPD plates, which were then incubated at 30°C for 24 h to allow the yeast cell to form colonies. When small colonies were formed, 10 ml 0.05% (w/v) TTC (separately sterilised), 0.5% (w/v) glucose and 1.5% (w/v) agar was poured on the top of the YPD agar plate. The plate was then incubated at 30°C overnight to stain the colonies. *S. cerevisiae* W-3 showed red and round-form colonies and *I. orientalis* KMBL 5774 showed pink or white and undulate-form colonies on the plate.

Analytical methods

For the analysis of alcohol contents during the wine fermentation, samples were collected and filtered through Whatman No. 1 filter paper. The filtrates were distilled to obtain distillates, which were cooled to 15°C and made up with distilled water to the same volume of the filtrate before the distillation. Alcohol content was assayed using a hydrometer based on the specific gravity of wine distillates and was expressed as % (v/v) at 15°C (see Caputi 1995).

Malic acid in wine was determined by HPLC with a Shodex RSpak KC-811 column (diam. 8 × 300 mm, Showa Denko KK, Kawasaki, Japan). The column was at 1 ml/min 40°C and 0.1% phosphoric acid was used as a mobile phase. Organic acids were detected with a refractive index detector.

Acetaldehyde and various alcohols were determined using GC with a HP-FFAP column (diam. 0.25 mm × 30 m) with temperature programming. An FID was used for detection.

The reducing sugar and total acid contents were determined by the AOAC method (Caputi 1995). Hue and intensity values of the wine were obtained from OD_{420}/OD_{520} and $OD_{420} + OD_{520}$, respectively. Hunter's value was determined using a vertical type spectrophotometer (Konica Minolta Holdings, Inc. CM-3600d, Tokyo, Japan).

Sensory evaluation of wine

Sensory evaluation of the wine was conducted according to 5-point hedonic scale. The panel was composed of 10 judges, (five females and five males) in the Department of Food Science and Technology, Kyungpook National University, Korea, who were sensitive at taste discrimination. Sensory scores were 5 (excellent), 3 (fair) and 1 (very poor). All the data were analysed with the SPSS (Statistics Package for the Social Science, version 12.0 for Windows) package to obtain averages and standard deviations. ANOVA was conducted to compare physicochemical characteristics and sensory properties according to each wine and to test the significance between average values of each measurement at $P < 0.05$ using Duncan's multiple range test.

Results and discussion

Fermentation characteristics of grape must by *I. orientalis* KMBL 5774

Wine fermentation by *I. orientalis* KMBL 5774 using Korean Campbell's Early grape must was carried out in order to investigate the possible application of the yeast strain to the wine brewing. During the fermentation, changes in the alcohol and reducing contents were assayed and compared with those obtained by *S. cerevisiae* W-3, one of the major wine yeast strains with industrial application for the wine brewing (Fig. 1). The strain W-3 showed a high and rapid alcohol production and reached the maximal level (7.8%, v/v) after 10 days of fermentation from the grape must with 14.8 °Brix of soluble solids. Most of the reducing sugar was consumed during the fermentation. However, KMBL 5774 showed a poor alcohol production rate and a low maximal level compared with those by W-3. The maximal level in the alcohol production was obtained after 15 days of fermentation, which was 5.8% (v/v) (Fig. 1a).

Total acid content in the wine fermented by the strain W-3 reached 0.78% after 15 days. However, the wine fermented by KMBL 5774 has less acid content (0.68%). The wine fermented by KMBL 5774 had less malic acid than that obtained by W-3: 0.31 mg/ml composed to 1.1 mg/ml.

Wine fermentation characteristics by the mixed culture of W-3 and KMBL 5774

The data obtained in preliminary experiments suggest that the strain KMBL 5774 is not suitable to use for

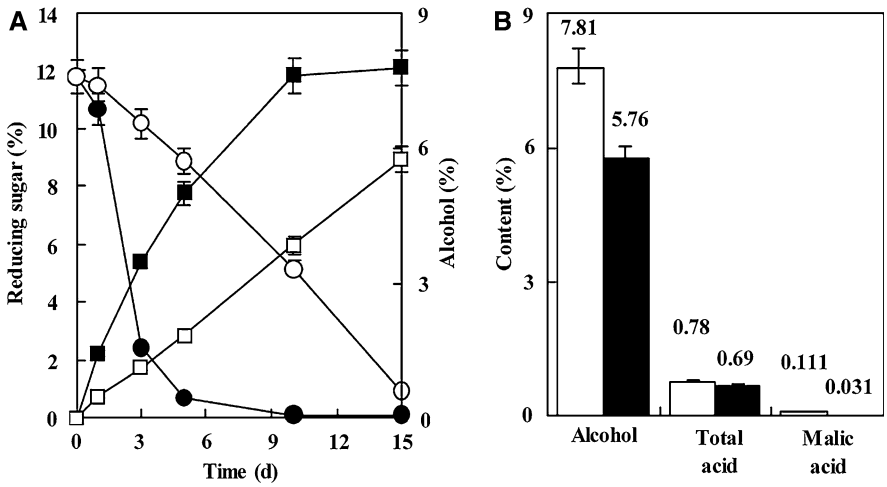

Fig. 1 Alcohol fermentation of grape must by *S. cerevisiae* W-3 (an industrial wine yeast) and *I. orientalis* KMBL 5774 (a malic acid-degrading yeast). Fermentation was carried out at 20°C for 15 days using the grape must containing 200 μg/ml potassium metabisulfite. Alcohol contents were expressed as % (v/V) and other contents were expressed as % (w/v). Panel a: Changes in the reducing sugar (•) and alcohol contents (■) by *S. cerevisiae* W-3 and the reducing sugar (○) and alcohol contents (□) by *I. orientalis* KMBL 5774. Panel b: Alcohol, total acid and malic acid contents in the wine fermented by *I. orientalis* KMBL 5774 (□) and *S. cerevisiae* W-3 (■)

the brewing of grape wine by itself but could be used for the degradation of malic acid in the wine. Therefore, we tested the effects of the co-fermentation with various inocula ratios (4:1, 1:1 and 1:4 based on the culture volume) of W-3 and KMBL 5774 on the fermentation characteristics. When 4:1 and 1:1 inocular ratio of W-3 and KMBL 5774 were used, patterns of alcohol production and reducing sugar consumption were similar to those obtained by W-3 alone. Use of 1:4 ratio of the two strains resulted in the prolonged fermentation time to reach the maximal alcohol production and sugar consumption, although they reached just a little lower level in the alcohol content than those obtained with other co-fermentations after 15 days (Fig. 2a).

During the co-fermentation with inocula ratio of 1:1 (v/v) of *S. cerevisiae* W-3 and KMBL 5774, each viable count was determined and compared with those obtained during the fermentation by W-3 alone (Fig. 2b). The viable count of KMBL 5774 increased from 6.9 log cfu/ml to 8.2 in a similar pattern to that of W-3 for the first 2 days of fermentation. However, it rapidly decreased to 7.5 $\log_{10}$ cfu/ml after 3 days and reached 6.8 $\log_{10}$ cfu/ml after 15 days of fermentation. However, the viable count of *S. cerevisiae* W-3 increased from 7.0 log cfu/ml to 8.4 for 7 days, which is the highest level during the fermentation. Since then, it has decreased and reached 7.5 log cfu/ml after 15 days of fermentation. Changes in the viable count of W-3 during the co-fermentation were almost the same as those obtained during the fermentation by W-3 only. Therefore, strain KMBL 5774 does not significantly influence the growth and alcohol fermentation by W-3 during the co-fermentation.

Physicochemical properties of the wine fermented by the mixed culture

After the fermentation, malic acid contents in the filtrate of the fermented mixture were determined using an HPLC (Fig. 3). The wine fermented by the strain W-3 alone had 1.1 mg malic acid/ml. When the mixed culture at 4:1(v/v) was used, malic acid content in the wine decreased to 0.61 mg/ml. Inocula ratios of 1:1 and 1:4 gave 0.33 and 0.32 mg of malic acid/ml.

Other physicochemical properties of the wine are shown in Table 1. The co-fermentation decreased the contents of acetaldehyde, 1-propanol, 2-butanol and isoamyl alcohol but increased the methanol content. Although no great differences were observed in the lightness of the wine, an increase in the redness and a

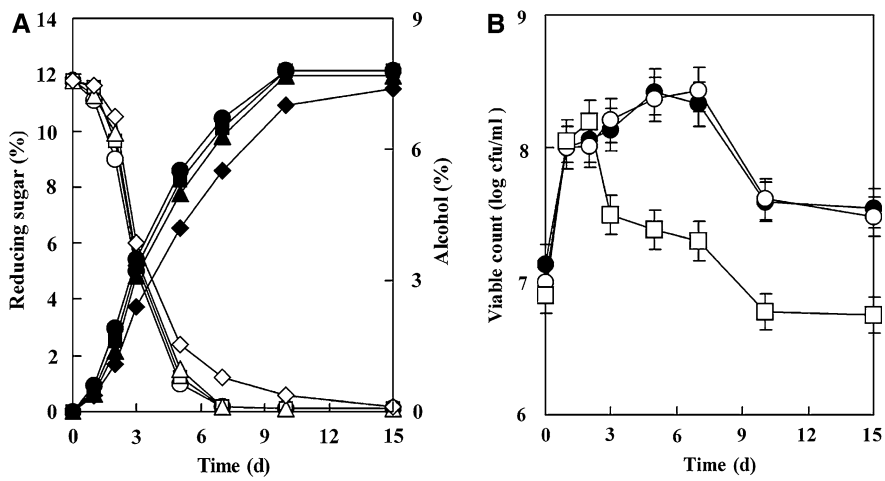

Fig. 2 Effects of the inocula ratio of *S. cerevisiae* W-3 and *I. orientalis* KMBL 5774 on the alcohol fermentation by their mixed culture. After W-3 and KMBL 5774 cells were inoculated into the grape must containing 200 μg/ml potassium metabisulfite. with the ratio of 1:0, 4:1, 1:1 and 1:4 based on their culture voulmes the fermentation was carried out at 20°C for 15 days. Panel a: Changes in the alcohol content (%, v/v) with inocular ratio of 1:0 (●), 4:1 (■), 1:1 (▲) and 1:4 (♦) and reducing sugar content (%, w/v) with inocular ratio of 1:0 (○), 4:1 (□), 1:1 (Δ) and 1:4 (◇). Panel b: Changes in the viable counts of W-3 (○) and KMBL 5774 (□) during co-fermentation with inocular ratio of 1:1 and viable counts during fermentation by *S. cerevisiae* W-3 alone as a control (●). Viable counts of the two yeasts were determined using the TTC staining described in the Material and method section

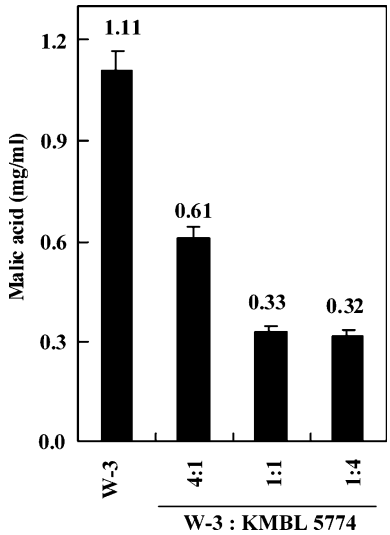

Fig. 3 Effects of the inocula ratio of *S. cerevisiae* W-3 and *I. orientalis* KMBL 5774 on the malic acid content in the wine fermented by their mixed culture. Inoculation of yeast cells and fermentation conditions were the same as those shown in Fig. 2

Table 1 Effects of the inocula ratio of *S. cerevisiae* W-3 and *I. orientalis* KMBL 5774 on some physicochemical properties of the wine fermented by their mixed culture

Inocula ratio (v/v) (W-3:KMBL 5774)	W-3 alone	4:1	1:1
Acetaldehyde (μg/ml)	52.3	36.5	32.9
Alcohols (μg/ml)			
Methanol	152	193	197
1-Propanol	30	23	22
2-Butanol	70	56	59
Isoamyl alcohol	195	154	152
Hunter's value			
L	39.8	38.7	39.7
a	14.4	22.3	25.2
b	−0.06	−0.07	−0.11
Sensory quality			
Color	2.4^{c}	2.6^{bc}	3.4^{a}
Flavor	1.6^{c}	2.4^{b}	2.4^{b}
Taste	2.2^{c}	3.2^{a}	3.4^{a}

Inoculation of yeast cells and fermentation conditions were the same as those shown in Fig. 2. After the fermentation, wines were prepared by filtration of the fermented mixture for the analysis of physicochemical properties. The mean scores with the same superscript within a row are not significantly different at 5% level at the Duncan's multiple range tests in the sensory evaluation

decrease in the yellowness were observed in the wine fermented by the mixed cultures compared to those in the wine fermented by W-3 alone. Sensory evaluation showed the highest score in the color, flavor and taste of the wine fermented with the inocula ration of 1:1 of W-3 and KMBL 5774. The same levels in the Hue and intensity of the wine (0.95 and 2.8, respectively) were obtained regardless of the inocula ratio, which is also the same as those obtained by W-3 alone (data not shown).

Issatchenkia orientalis was first isolated as a yeast with acid and ethanol tolerance (Okuma et al. 1986) and as one of the indigenous yeasts present in the wine (Clemente-Jimenez et al. 2004). *Issatchenkia orientalis* KMBL 5774 isolated from Korean wine pomace can degrade malic acid (Seo et al. 2007). However, not much information is available thus far on the function of *I. orientalis* in the brewing of wine. In this paper, *I. orientalis* KMBL 5774 could be important in decreasing malic acid content in the wine and therefore could be useful due to the wine industry by this attribute.

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