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ORIGINAL ARTICLE

Degradation of malic acid in wine by immobilized *Issatchenkia orientalis* cells with oriental oak charcoal and alginate

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Keywords
degradation, *Issatchenkia orientalis*, malic acid, oak charcoal, wine, yeasts.

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Abstract

Aims: To test degradation of malic acid content in wine by immobilized *Issatchenkia orientalis* KMBL 5774 cells recently isolated from Korean wine pomace as a malic acid-degrading yeast.

Methods and Results: *I. orientalis* KMBL 5774 cells were immobilized using a mixture of oriental oak (*Quercus variabilis*) charcoal with sodium alginate. When the immobilized yeast cells were observed on a scanning electron microscope, cells were efficiently immobilized on the surface area of the charcoal. A Korean wine containing a high level of malic acid was treated with the immobilized yeast cells. The HPLC analysis of the malic acid content in the treated wine showed the malic acid content was reduced to 0·75 mg ml^{-1} after treatment from the original content of 8·96 mg ml^{-1}, representing 91·6% of the malic acid was degraded during the treatment.

Conclusions: The immobilization of the malic acid-degrading yeasts with oriental oak charcoal and sodium alginate is useful for degradation of malic acid in wines containing a high level of malic acid with no significant increase in other acids.

Significance and Impact of the study: Malic acid is sometimes detrimental to the quality of wines when present at high concentrations in some varieties. The immobilized *I. orientalis* KMBL5774 cells appear to be a promising candidate in view of developing biotechnological methods for reduction of malic acid contents in wine.

Introduction

Consumption of wine in Korea has greatly increased in the past 20 years. However, the majority of the wines consumed in Korea are dependent upon imported wines from other countries. It has shown that the Korean domestic wines are limited to only 19·6% of the whole wine consumption in 2006. A survey revealed that they were low in quality because of sour taste derived from too much acid contents and low flavours compared with imported wines (Lee *et al.* 2004). Korean domestic wines are fermented using Campbell's Early grapes (*Vitis labruscana* cultivar. Campbell's Early), which has occupied about 70% of the total grape production in Korea (Yook *et al.* 2007). The grape contained a low level of sugar (13–15%) and a high level of malic acid content (4–16 mg ml^{-1}) enough to negatively affect the wine quality, especially when it was cold during the summer season (Park *et al.* 2004).

Malic acid is one of the major organic acids together with tartaric acid in the grape juice and wine. Grapes

generally contain 3–7 mg ml^{-1} of tartaric acid and 1–10 mg ml^{-1} of malic acid. Malic acid is sometimes detrimental to the quality of wines when present at high concentrations in some varieties (Ruffner 1982; Pretorius 2000). It contributes to the acidic taste of wine and serves as a substrate for contaminating lactic acid bacteria that can cause wine spoilage after bottling (Ruffner 1982; Henick-Kling 1993). Its levels in wine grapes are of a great concern of wine-makers to improve the sensory quality of wines (Thornton and Rodriguez 1996). It is therefore essential to remove excess malic acid from the wine to ensure the physical, biochemical and microbial stabilities and quality of the wine (Pretorius 2000). However, the major wine yeast strains of *Saccharomyces* sp. do not degrade malic acid effectively during alcoholic fermentation (Volschenk *et al.* 1997). This is attributed to the poor uptake of malic acid by simple diffusion (Ansanay *et al.* 1996) and the low substrate affinity of their malic enzyme in *Saccharomyces* sp. (Fuck *et al.* 1973). Production of malic acid by some strains of *Saccharomyces cerevisiae* was also observed during the wine fermentation (Schwartz and Radler 1988).

Microbial cell immobilization is an attractive area in the fermentation and wastewater treatment owing to its additional technical and economical advantages compared with the free cell system (Bardi *et al.* 1996). It can offer many advantages for promoting functional efficiency of the cells such as high cell concentration in the reactor, easy separation of the immobilized cells in the settling tank, short lag period of fermentation and stability of the fermentation system compared with free-living cells (Bardi *et al.* 1996). Recently, cell immobilization has been also shown to improve significantly the tolerance to the ethanol stress of yeast and bacterial strains (Zhou *et al.* 2008). A number of studies have been published on the alcohol production with immobilized yeast cells by batch (Kolpakchi *et al.* 1980) as well as continuous fermentation (Linko and Linko 1981). Wine yeast cells were also entrapped in corn starch gel or immobilized on cork pieces for the purpose of wine fermentation (Tsakiris *et al.* 2010). One of the promising methods for immobilization of cells is to encapsulate or entrap microbial cells using polymers. Many natural materials and synthetic polymers such as polyacrylamide, agar, polyvinyl alcohol (PVA), sodium or calcium alginate have been studied extensively to apply to microbial cell immobilization. The most efficient and popular methods for cell immobilization are the entrapment of cells in sodium or calcium alginate (Hamedi *et al.* 2005; Kar *et al.* 2008) and starch gel (Panagiotis *et al.* 2008). This technique is not only simple and inexpensive but also nontoxic because the polymer is a natural polysaccharide, which is abundant in nature and commercially available with a cheap price (Yu *et al.* 2004).

Previously, we have isolated *I. orientalis* KMBL 5774, a malic acid-degrading yeast from Korean wine pomace (Seo *et al.* 2007). Cofermentation of grape must by the mixed culture of the yeast strain KMBL 5774 and an industrial wine yeast strain resulted in a great reduction of malic acid content in wine (Kim *et al.* 2008). In this study, the yeast cells were immobilized using oriental oak charcoal and sodium alginate to test degradation of malic acid content in wine by immobilized *I. orientalis* KMBL 5774 cells.

Materials and methods

Strains and culture conditions

The yeast strain degrading malic acid, *I. orientalis* KMBL5774, was isolated from Korean wine pomace and has been described in detail, previously (Seo *et al.* 2007; Kim *et al.* 2008). Yeast cells were grown at 30°C for 2 days in YPD liquid media (1% yeast extract, 2% Bacto-peptone, 2% glucose) with shaking at 150 rev min^{-1} for the growth. To prepare yeast cells for immobilization, cells were grown in YNB-malic acid liquid media composed of 0·17% yeast nitrogen base w/o amino acid and ammonium sulfate, 0·5% ammonium sulfate and 2% malic acid as a sole carbon and energy source (Seo *et al.* 2007).

Preparation of charcoals

Charcoals were prepared by the method of Saito and Arima (2002) using wood of various Korean trees such as *Acer mono* Max (maple), *Quercus variabilis* Blume (oriental oak), *Prunus sargentii* Rehder (wild cherry) and *Pinus densiflora* Sieb. et Zucc (Korean red pine). Preliminary charcoalized wood of the trees prepared at 300–400°C for 3 h in a furnace was used as starting material. The samples cut into $20 \times 20 \times 50$ mm (L direction) were heated at a rate of 10°C min^{-1} at a pressure of 10–20 Pa and pyrolysed at 800°C for l h under Ar gas in an electric furnace (Shinsei Electric Co. Ltd. SDS46-4, Okayama, Japan). The charcoal prepared at 800°C for l h showed the best moisture sorption behaviour in a preliminary experiment. After the treatment, the charcoals were cooled down to room temperature at a rate of 30°C min^{-1}.

Immobilization of yeast cells

Issatchenkia orientalis KMBL 5774 cells were immobilized using oriental oak charcoal powder and sodium alginate by the method of Son *et al.* (2003). The oriental oak charcoal powder was prepared by passing through a 100

mesh sieve after grinding. Charcoal suspension was prepared by continuous shaking a mixture of 1·2 g of oriental oak charcoal powder and 180 ml of 0·85% NaCl solution. Sodium alginate (4 g; Fluka AG, Geneva, Switzerland) was added to the charcoal suspension and mixed well, which was sterilized in an autoclave at 121°C for 15 min. The sterilized suspension was cooled down to 45°C. Separately, yeast cells grown in YNB-malic acid media were harvested by centrifugation and washed with sterilized 0·85% NaCl solution to remove residual media components. Yeast cells (8 g wet weight cells) were resuspended in 20 ml of sterilized 0·85% NaCl solution. The cell suspension was warmed up to 45°C in water bath and mixed with the charcoal suspension. The mixture was then extruded through a 10-ml syringe fitted with an 18-gauge needle into 3% $CaCl_2$ solution at 4°C, which was kept in the same solution at 4°C for 24 h to be stabilized. The stabilized yeast cells were washed three times with sterilized distilled water before use.

Scanning electron microphotograph (SEM)

Samples were fixed at 24°C for 60 min with 2·5% glutaraldehyde in 0·1 mol l^{-1} sodium cacodylate buffer (pH 7·2) (Sigma Aldrich, St Louis, USA). The fixed samples were dehydrated with a serial concentration of ethanol and then dried on a critical point drier (Hitachi Co. HCP-2, Tokyo, Japan). The dried samples were coated with gold by the method of Knutton (1995) and finally examined using a scanning electron microscope (Hitachi Co. S-4300).

Treatment of wine with immobilized yeast cells

A Korean wine prepared using Campbell's Early grapes (*Vitis labruscana* cultivar. Campbell's Early) was obtained from the commercial market. Physicochemical properties of the wine were as following: alcohol 12·0% (v/v), pH 3·53, total acid 0·46%, malic acid 8·96 mg ml^{-1}, reducing sugar 1·62% and acetic acid 91 mg ml^{-1}. Immobilized yeast cells (2·5 g) were added into 1 l of the wine, which was incubated at 30°C for 30 h with shaking at a speed of 150 rev min^{-1}. During the incubation, samples were collected every 6 h and centrifuged at 10 000 ***g*** to remove immobilized yeast cells from the wine. After centrifugation, the supernatant was filtered through a 0·45-μm microfilter and used for the analysis of malic acid content and other assays.

Analytical methods

Malic acid contents in wine were determined using an HPLC (Waters Co. 600E, Milford, MA) attached with a Shodex RSpak KC-811 column (Φ 8·0 × 300 mm, Showa Denko K. K., Japan). The column was at 0·8 ml min^{-1}, 40°C, and 0·1% phosphoric acid was used as a mobile phase. Organic acids were detected with a refractive index detector (RI; Waters Model 410, Milford, MA, USA). For the analysis of alcohol contents, the filtrates were distilled to obtain distillates, which were cooled down to 15°C and filled up with distilled water to the same volume of the filtrates before the distillation. Alcohol contents were assayed using a hydrometer based on the specific gravity of wine distillates and was expressed as % (v/v) at 15°C by the AOAC method (Caputi 1995). Reducing sugar contents were determined using dinitrosalicylic acid (Dubois *et al.* 1956). Total acid contents were determined using a 0·1 N NaOH solution and expressed as % malic acid (Caputi 1995). Hue and intensity values of the wine were expressed as OD_{420}/OD_{520} and $OD_{420} + OD_{520}$, respectively, by the method of Auw *et al.* (1996) after optical density of the filtrates was measured at 420 and 520 nm using a spectrophotometer (Shimadzu Co. UV-1601, Kyoto, Japan).

Results

Immobilization of *I. orientalis* cells

Cells of a malic acid-degrading yeast, *I. orientalis* KMBL 5774, were immobilized using oriental oak charcoal and alginate to test possible applications of the yeasts to reduce malic acid content in wine. Four different charcoals were prepared using wood of *A. mono* Max (maple), *Q. variabilis* Blume (oriental oak), *P. sargentii* Rehder (wild cherry) and *P. densiflora* Sieb. et Zucc (Korean red pine). When they were observed on a scanning electron microscope, they showed very different features in their porosity and pore size (Fig. 1). In the porosity, *P. sargentii* charcoal (C) showed the most thinly scattered and sparse pores among them followed by *Acer mono* charcoal (A). Charcoals made from *Q. variabilis* (B) and *P. densiflora* trees (D) exhibited high porosity. *P. densiflora* charcoal showed the biggest pore size (9·58–13·51 μm) followed by *Q. variabilis* charcoal (4·58–8·06 μm). The pore size of *A. mono* and *P. sargentii* charcoals was relatively small compared with that of *P. densiflora* and *Q. variabilis* charcoals. Pores in the charcoal can provide microbial cells with more chances to be lodged in them and also affect positively the stabilization of the immobilized cells. In this case, high porosity and suitable size of the pores could provide chances for more and better insertion of the cells (Guo and Lua 1998). Therefore, *Q. variabilis* charcoal with high porosity and 4·58–8·06 μm in pore size was selected for the immobilization of the yeast cells, whose size is generally known to be 4–5 μm (Kurtzman 1998).

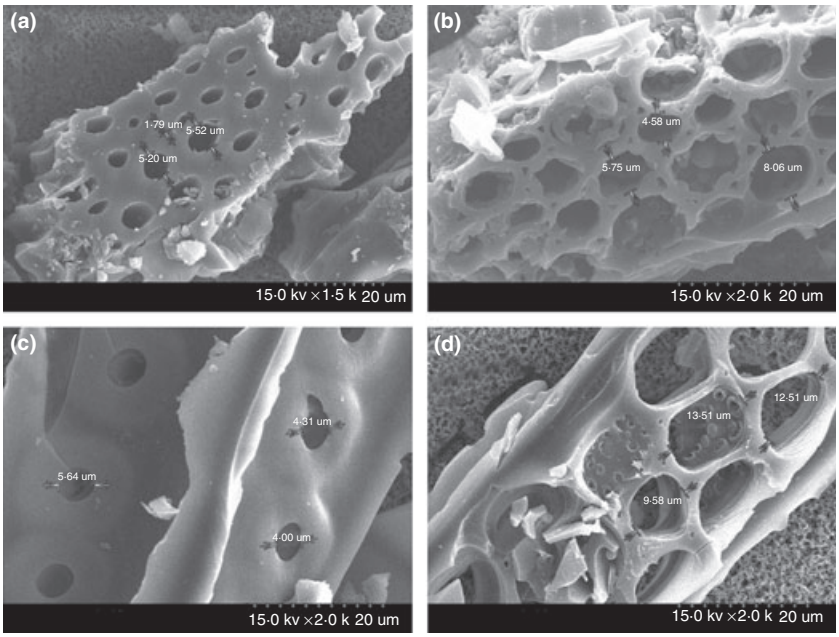

Figure 1 Scanning electron microphotographs of various charcoals. (a) *Acer mono* Max (maple), (b) *Quercus variabilis* Blume (oriental oak), (c) *Prunus sargentii* Rehder (wild cherry), (d) *Prinus densiflora* Sieb. et Zucc (Korean red pine).

The shape and surface structure of the immobilized yeast cells are shown in Fig. 2. The immobilized yeasts showed a thread-like shape (A). When its surface structure was observed on a scanning electron microscope, many yeast cells were observed to adhere to the surface area of the charcoal by alginate (B). Activated carbons are used in a variety of applications including as an adsorbent and catalyst support because of their high adsorptive capacities, adequate pore size distributions and relatively high mechanical strengths (Guo and Lua 1998). Alginate has been shown to be very efficient for microbial cells immobilization together with activated carbons (Son *et al.* 2003). Its concentration was known to determine mass transfer phenomena of nutrients and biomass, which in turn are directly related to the cell growth, mechanical stability of the immobilized cells and maintain a high production yield (Yu *et al.* 2004; Hamedi *et al.* 2005).

Degradation of malic acid by immobilized yeast cells

The immobilized *I. orientalis* cells were treated in a Korean wine containing a high level of malic acid content for 30 h. After the treatment, organic acids in the wine were separated using an HPLC and compared with those in the wine before the treatment (Fig. 3). In the original wine before the treatment as shown in Fig. 3a, malic acid was the most abundant organic acid (8·96 mg ml^{-1}), and lactic acid was the second (4·18 mg ml^{-1}). A relatively low level of tartaric acid content (1·75 mg ml^{-1}), which was the 4th highest level, was obtained compared with that of two major organic acids described above. The third and fifth highest peaks at 8·362 and 6·263 min of retention time are yet to be identified. The treatment of immobilized yeast cells resulted in a dramatic reduction in the malic acid content in wine, which was only 0·79 mg ml^{-1}. The amount of an unidentified acid with 8·617 min of retention time also reduced. However, the content of the other unidentified acid with 6·315 min of retention time rather increased. The corelation of the changes in the content of three acids has not been elucidated yet.

To confirm the reduction in malic acid content in the wine treated with the immobilized yeast cells, we have repeated the experiment and assayed changes in the malic acid content more carefully during the treatment (Fig. 4). Total acid content was 0·46% in the wine before the treatment. The content was reduced to 0·41% for the first 6 h and reached the lowest level (0·38%) after 24 h, which has showed no change for another 6 h (Fig. 4a). A rapid reduction in the malic acid content was also observed during the early stage of the treatment, and the reduction was slowed as the treatment time was prolonged. The malic acid content was reduced from 8·96 to 3·37 mg ml^{-1} after 6 h and to 1·08 mg ml^{-1} after 12 h. It finally reached 0·75 mg ml^{-1} after 30 h of treatment, which represents 91·6% degradation of the malic acid content compared with that in the wine before the treatment. However, a slight reduction in the tartaric acid level was observed during the treatment of the wine. Its content was reduced from 1·75 to 1·47 mg ml^{-1} after the treatment, which was 84·0% level in the wine before treatment (Fig. 4b). The data shown here suggest that *I. orientalis* KMBL 5774 cells immobilized using the oriental oak charcoal and sodium

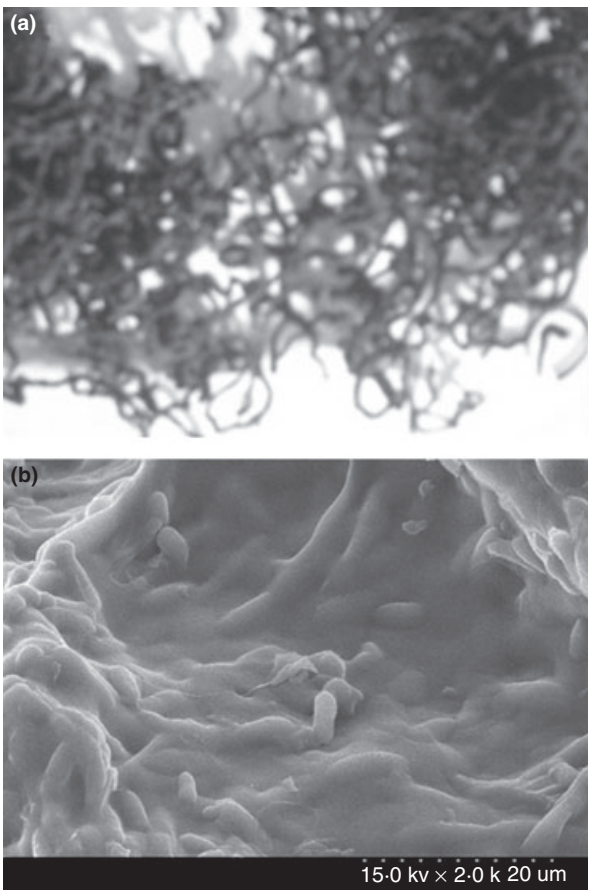

Figure 2 Shape (a) and scanning electron microscopic observation (b) of the immobilized *Issatchenkia orientalis* KMBL 5774 cells. The yeast cells were immobilized with oriental oak charcoal and sodium alginate.

alginate could efficiently degrade malic acid in wine, which is highly specific to the malic acid.

Effects of immobilized cells on the properties of wine

Effects of the treatment of the immobilized cells were investigated on the properties of the wine such as alcohol and reducing sugar contents as well as colour Hue and intensity values of the wine (Fig. 5). Although the alcohol content was reduced during the treatment, the reduction ratio was not very high. Its content decreased from 12·0 to 10·1% (v/v) after 30 h of treatment. However, the content of reducing sugar was dramatically reduced to 0·20%, which is about 12·3% level of the content in the wine before treatment (1·62%) (Fig. 5a). *I. orientalis* KMBL 5774 was isolated from a Korean wine pomace as a yeast strain degrading malic acid as a sole carbon and energy source. It was also shown that the yeast cells could degrade malic acid regardless of the presence of assimilable sugar, suggesting that the strain belongs to K (+) group of malic acid utilizing yeasts (Seo *et al.* 2007). Therefore, it was thought the reduction in the malic acid content during the treatment in wine is attributed to the fact that the immobilized yeast cells were grown using the residual reducing sugar together with the malic acid in the wine. A slight decrease in the colour Hue value was observed together with a significant reduction in the intensity value during the treatment of the immobilized yeast cells (Fig. 5b). The colour Hue and intensity values are important factors in the evaluation of the wine quality. Generally, the Hue value is about 0·5 for red wines before sufficient ageing and 1·0 or above for over-oxidized red wines (Boido *et al.* 2006). In this study, decrease in the colour Hue and intensity values was observed, which may negatively affect the quality of wine. The mechanism for their reduction is to be elucidated to maintain the colour in the wine during treatment of the immobilized cells of malic acid-degrading yeast.

Discussion

A malic acid-degrading yeast, *I. orientalis* KMBL 5774, has been recently isolated from Korean wine pomace, and its possible application has been studied to cofermentation with a wine yeast for reduction in malic acid content in wine. In this study, cells of the strain KMBL 5774 were immobilized using the mixture of oriental oak (*Q. variabilis*) charcoal with sodium alginate. The oriental charcoal showed the most suitable features such as porosity and pore size for immobilization of the cells among various charcoals made from maple, wild cherry and Korean red pine tree wood. A Korean wine containing high level of malic acid was treated with the immobilized yeast cells with oriental oak charcoal and alginate. The HPLC analysis of the malic acid content in the treated wine showed a rapid and dramatic reduction in the malic acid content. The malic acid content was reduced to 0·75 mg ml^{-1} after treatment, in which the degradation ratio of malic acid was 91·6% compared with that before treatment. Most of the reduction occurred during 12 h of the treatment. However, a slight decrease was observed in the content of tartaric acid, another major organic acid together with malic acid in wine.

There have been a great number of studies to reduce the excessive malic acid content in wine. The malo-lactic acid fermentation (MLF), the conversion of malic acid to lactic acid and carbon dioxide by strains of lactic acid bacteria, is the principal biological method of reducing residual malic acid contents in wine. Strains of the lactic acid bacteria including *Oenococcus oeni* as well as more recently *Lactobacillus plantarum* and *Lactobacillus casei* are used to perform MLF in wine (Henick-Kling 1993; Agouridis *et al.* 2005, 2008). Degradation of malic acid in

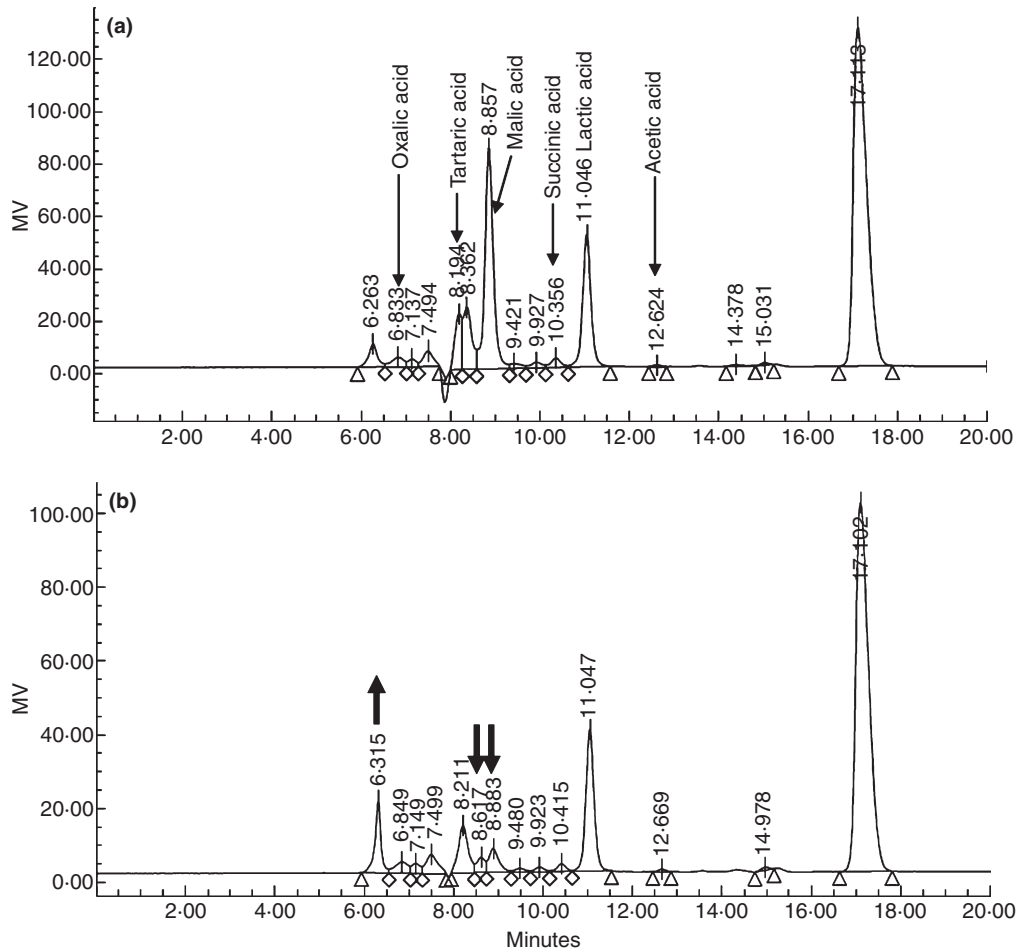

Figure 3 HPLC chromatograms of organic acids in the wine before (a) and after (b) treatment with the immobilized yeast cells. The wine was treated with immobilized yeast cells at 30°C for 30 h.

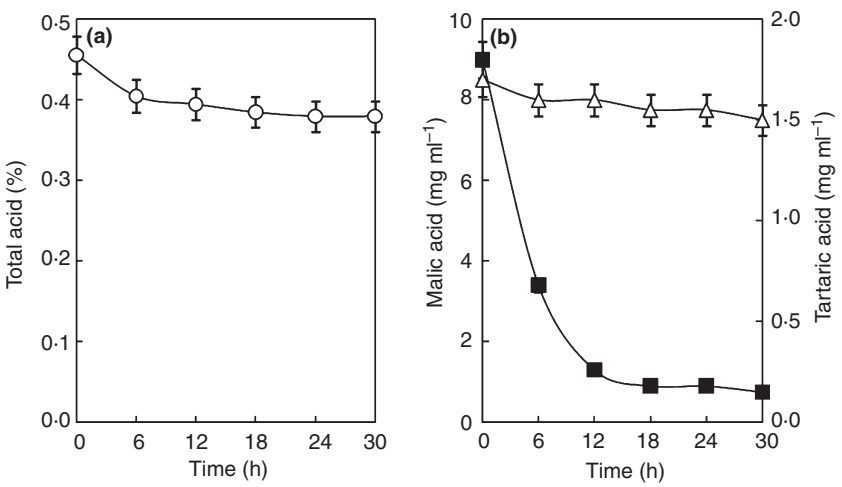

Figure 4 Changes in the total acid (a), malic and tartaric acid contents (b) during the treatment of wine with the immobilized yeast cells. ○: total acid, ■: malic acid, △: tartaric acid.

wine by yeasts has also attempted. Among the yeasts reported to degrade extracellular malic acid are *Zygosaccharomyces bailii*, *Candida sphaerica*, *Hansenula anomala*, *I. orientalis*, *Pachysolen tannophilus*, *Pichia stipitis* and *Schizosaccharomyces* sp. such as *Schizosaccharomyces malidevorans* and *Schizosaccharomyces pombe* (Gallander 1977;

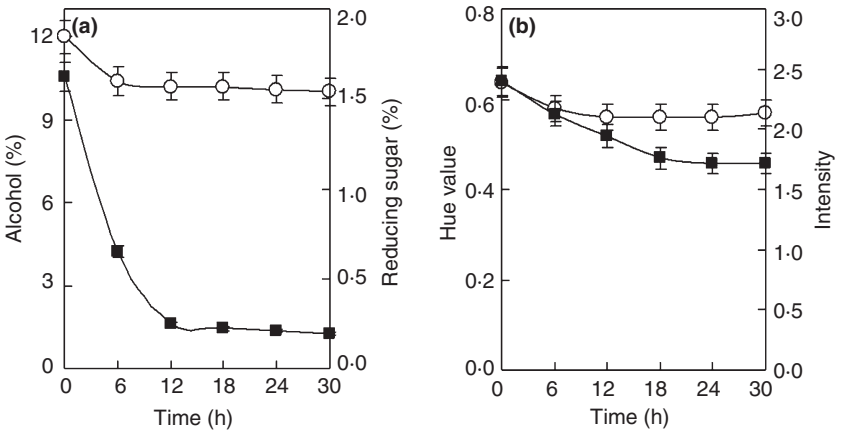

Figure 5 Changes in the alcohol (○) and reducing sugar (■) contents (a) as well as the colour Hue (○) and intensity (■) values (b) during the treatment of wine with the immobilized yeast cells.

Baranowski and Radler 1984; Rodriguez and Thornton 1989; Corte-Real and Leao 1990, 1992; Rodriquez and Thornton 1990; Thornton and Rodriguez 1996; Seo *et al.* 2007; Kim *et al.* 2008). *S. pombe* has been studied most intensively as a means of reducing wine acidity. However, *Schizosaccharomyces* sp. produces off-flavours and aromas in the wine (Magyar and Panyik 1989; Rodriquez and Thornton 1990). This characteristic has been the major barrier to the use of these yeasts in winemaking, although they are the best metabolizers of malic acid in grapes (Gallander 1977; Snow and Gallander 1979). To solve the problem in using *Schizosaccharomyces* sp., several studies including partial fermentation of *S. pombe* (Snow and Gallander 1979) and use of immobilized *S. pombe* cells to minimize the contact with the wine (Magyar and Panyik 1989; Rosini and Ciani 1993) have been employed. However, none of these approaches have been entirely successful for their industrial application to winemaking thus far. The immobilized cells of *I. orientalis* KMBL 5774 using oriental oak charcoal and alginate prepared in this study could greatly reduce the malic acid amount in a wine. Therefore, it was proposed that the immobilization of *I. orientalis* cells could be an alternative way for degradation of malic acid in wines.

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