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Degradation of Malic Acid by *Issatchenkia orientalis* KMBL 5774, an Acidophilic Yeast Strain Isolated from Korean Grape Wine Pomace

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Several yeast strains degrading malic acid as a sole carbon and energy source were isolated from Korean wine pomace after enrichment culture in the presence of malic acid. Among them, the strain designated as KMBL 5774 showed the highest malic acid degrading ability. It was identified as *Issatchenkia orientalis* based on its morphological and physiological characteristics as well as the nucleotide sequences of the internal transcribed spacer (ITS) I-5.8S rDNA-ITS II region. Phylogenetic analysis of the ITS I-5.8S rDNA-ITS II sequences showed that the KMBL 5774 is the closest to *I. orientalis* zhuan 192. Identity of the sequences of the KMBL 5774 was 99.5% with those of *I. orientalis* zhuan 192. The optimal pH of the media for the growth and malic acid degradation by the yeast was between 2.0 and 3.0, suggesting that the strain is an acidophile. Under the optimized conditions, the yeast could degrade 95.5% of the malic acid after 24 h of incubation at 30°C in YNB media containing 2% malic acid as a sole carbon and energy source.

Keywords: acidophilic yeast, grape, malic acid degradation, *Issatchenkia orientalis*

Malic acid and tartaric acid are the most abundant organic acids in grapes and grape wines. Must, obtained from wine grapes in the process of winemaking, generally contains 3-7 mg/ml of tartaric acid and 1-10 mg/ml of malic acid (Ruffner, 1982). These two acids not only contribute to 70-90% of the organic acids in the grape juice and wine but also significantly influence the sensory properties and qualities of wines (Beelman and Gallander, 1979; Ruffner, 1982; Henick-Kling, 1993; Radler, 1993; Gao and Fleet, 1995). The production of premium wines depends on the enologist's skill to accurately adjust wine acidity to obtain a balanced wine with optimum flavour and colour profile (Volschenk *et al.*, 1997, 2001). Therefore, their levels in wine grapes are of a great concern of wine makers and researchers to improve the sensory quality of wines (Gao and Fleet, 1995; Thornton and Rodriguez, 1996).

Malic acid, one of the most abundant organic acids together with tartaric acid in the grape, is detrimental to the quality of wines because of its too much content in some varieties (Volschenk *et al.*, 1997, 2001). Malic acid contributes to the acidic taste of wine and serves as a substrate for contaminating lactic acid bacteria that can cause wine spoilage after bottling. It is therefore essential to remove excess malic acid from the wine to ensure the physical, biochemical and microbial stability and quality of the wine (Delcourt *et al.*, 1995; Pretorius, 2000). The malic acid content is depending on the grape variety and the climate of the regions where they were grown (Ruffner, 1982; Volschenk *et al.*, 2001). Several grape varieties including Campbell's Early grape, which is the major grape variety in Korea, have been reported to contain high amount of malic acid enough to affect negatively the quality of wine (Gallander, 1977). Grapes grown in the cooler regions contain higher level of malic acid than those grown in the warmer regions. Excessive amounts of malic acid up to 15-16 mg/ml have been found in grapes at harvest time during exceptionally cold summers in cool viticultural regions (Gallander, 1977). However, wine yeast strains of *Saccharomyces* sp. routinely used for the wine fermentation in general do not degrade malic acid effectively during alcoholic fermentation (Volschenk *et al.*, 1997; Subden *et al.*, 1998). The inefficient degradation of malic acid by *S. cerevisiae* is ascribed to the slow uptake of malic acid by simple diffusion (Baranowski and Radler, 1984; Ansanay *et al.*, 1996; Volschenk *et al.*, 1997) and the low substrate affinity of its malic enzyme (K_m=50 mM) (Fuck *et al.*, 1973).

Reduction of malic acid in the wines has been attempted in several other ways. The malo-lactic 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* and more recently *Lactobacillus plantarum* are used to perform MLF in wine (Wibowo *et al.*, 1985; Henick-Kling, 1993). However, MLF is one of the most difficult processes in winemaking despite the availability of commercial preparations of the strains (Thornton and Ridriguez, 1996). Various yeast strains have been investigated as alternative agents for malic acid degradation in wine. Malic acid is converted to ethanol by several yeasts; thus, the reduction

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in acidity is more acute than in MLF wines. There have been several reports on the yeast strains degrading extracellular malic acid including *Candida sphaerica* (Corte-Real *et al.*, 1989), *Hansenula anomala* (Corte-Real and Leao, 1990, 1992), *Pachysolen tannophilus* (Rodriguez and Thornton, 1990), *Pichia stipitis* (Rodriguez and Thornton, 1990), *Schizosaccharomyces malidevorans, Schizosaccharomyces pombe* (Rankine, 1966; Benda and Schmitt, 1969; Gallander, 1977; Munyon and Nagel, 1977; Snow and Gallander, 1979) and *Zygosaccharomyces bailii* (Baranowski and Radler, 1984; Taillandier and Strehaiano, 1991). Among the yeast strains degrading malic acid, *S. malidevorans* and *S. pombe* are the strains, which have been studied most intensively as a means of reducing wine acidity (Rankine, 1966; Benda and Schmitt, 1969; Gallander, 1977; Munyon and Nagel, 1977; Snow and Gallander, 1979). However, *Schizosaccharomyces* sp. produces off-flavors and aromas in the wine (Rodriguez and Thornton, 1989, 1990). This characteristic has been the major barrier to the use of these yeasts in winemaking (Rankine, 1966; Gallander, 1977; Munyon and Nagel, 1977; Snow and Gallander, 1979). In order to solve the problem in using *Schizosaccharomyces* sp., several studies including isolation of mutant strain producing low H_2S (Rankine, 1966), mixed fermentation with *S. cerevisiae* (Snow and Gallander, 1979), immobilization of the cells to minimize the contact with the wine (Magyar and Panyik, 1989; Rosini and Ciani, 1993; Yokotsuka *et al.*, 1993), etc. have been employed. However, none of these approaches have been entirely successful for the industrial application of winemaking thus far.

The aim of this work was to isolate and characterize a malic acid-degrading yeast strain which can be applied to the fermentation of grape must containing high level of malic acid. Several yeast strains with malic acid degradability were isolated from Korean grape wine pomace. A strain with the highest degradability was selected and applied to the fermentation of Campbell's Early grape must with high level of malic acid content.

Materials and Methods

Media and culture conditions

A yeast strain, *Issatchenkia orientalis* KMBL5774, degrading malic acid was isolated from a Korean wine pomace using YNB-malic acid agar plates (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate, 0.5% ammonium sulfate, 2% malic acid, 2% agar). YPD broth (1% yeast extract, 2% bacto-peptone, 2% glucose) was used for pre-culture of the yeast. For the test of malic acid degradation, YNB liquid media containing 2% malic acid as the sole carbon and energy sources was used. Cells were stored at -70°C in the presence of 15% glycerol.

Isolation of yeasts degrading malic acid

Wine pomaces were collected from several wineries in Korea for the isolation of yeasts. Yeast cells degrading malic acid in the pomace were enriched at 30°C for 3 days in YNB media containing 2% malic acid as the sole carbon and energy sources. After the enrichment culture, the cultures were diluted with 0.9% NaCl solution. The diluted cultures were spread on YNB-malic acid plates, which were incubated at 30°C for 2 days. Colonies were picked and grown on the YNB-malic acid plates for isolation of single colony. All the strains were tested for their growth and malic acid degradation in YNB-malic acid liquid media. The yeast strain showing the highest malic acid degradability was selected for further studies.

Sequence analysis of yeast rDNA internal transcribed spacer (ITS) region

For the analysis of ITS I-5.8S rDNA-ITS II sequences of the isolated yeast, DNA fragments containing the regions between 3' terminus of 18S rDNA and 5' terminus of 25S rDNA were amplified using PCR. Yeast genomic DNA was extracted using the Dneasy Tissue Kit (Qiagen, USA) following the instructions of the manufacturer. DNA was eluted with 100 μl of AE buffer from the same kit. PCR was carried out in 20 μl containing the genomic DNA as the template. Forward primer; 5'-CGCGGATCCGTAGGTGAACCTGCGG and reverse primer; 5'-CGCGGATCCCTCCGCTTATTGAT ATG were used together as PCR primers for the amplification (Granchi *et al.*, 1999; Park *et al.*, 1999; Torija *et al.*, 2001). The PCR mixture consisted of 1 μg of template DNA, 100 pmol of each primer, 1 U of Taq DNA polymerase, 0.25 mM each dNTP, 10 mM Tris·HCl buffer (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$. The PCR cycle program for the DNA amplification was comprised of one cycle of 94°C for 3 min, 31 cycles of 94°C for 1 min, 45°C for 2 min, 72°C for 1.5 min, and finally one cycle of 72°C for 10 min. After the amplification, the PCR products were purified using a Dynabeads PCR Clean Up Kit (Dynal Biotech ASA, Norway). Sequencing reaction of the PCR products was performed using a Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). The sequencing reaction products were resolved on an automated DNA sequencing system (Applied BioSystems model 3730XL, USA).

Yeast identification

The yeast identification was performed according to the method of Kurtzman (1998) based on its morphological and physiological characteristics as well as nucleotide sequences of its ITS I-5.8S rDNA-ITS II region.

Phylogenetic analysis

Nucleotide sequences of the ITS I-5.8S rDNA-ITS II region were compared with those available in the GenBank database by using the BLAST method to determine their approximate phylogenetic affiliation and their sequence similarities at the National Center for Biotechnology Information, USA (Altschul *et al.*, 1997; http://ncbi.nlm.nih.gov/BLAST). The sequences of the related taxa were acquired from the same web site. Nucleotide sequences were initially aligned using the CLUSTAL X program (Thompson *et al.*, 1997) and then manually adjusted. Distance matrices were calculated and phylogenetic tree for the data set was created according to the Kimura two-parameter model (Kimura, 1980) and neighbor-joining method (Saitou *et al.*, 1987) by using the phylip (version 3.67) software packages obtained from the web site (http://evolution.genetics.washington.edu/phylip.html). One thousand bootstraps were performed to assign confidence levels to the nodes in the tree.

Determination of malic acid content
The residual malic acid content in the yeast culture in the YNB-malic acid broth was determined by a colorimetric method (Goodban and Stark, 1957). One ml of culture supernatant, obtained by centrifugation of the culture broth, was mixed well with 0.1 ml of 1% 2,7-dihydroxynaphtalene in conc. H_2SO_4 and 6 ml of conc. H_2SO_4. After the mixture was incubated at room temperature for 20 min, the increase in optical density was measured at 390 nm using a Spectrophotometer (Shimadzu UV161, Japan). The amount of the malic acid was calculated from the optical density using malic acid as a standard.

Results

Isolation and characterization of malic acid-degrading yeasts
Several malic acid-degrading yeasts were isolated from Korean grape wine pomace using YNB-malic acid agar plates containing 2% malic acid as the sole carbon and energy sources after enrichment culture at 30°C for 3 days in YNB-malic acid liquid media. After picked as a single colony on agar plates, all of them were tested for their malic acid degradability in YNB-malic acid liquid media. Among them, the strain designated KMBL 5774 exhibited the highest level in the growth and malic acid degradability, which was about 88%. It was observed to be elongate and to reproduce by budding on a microscope. The strain was selected as a malic acid degrading yeast for further studies.

Various morphological and physiological characteristics of the strain KMBL 5774 were investigated for its identification (Table 1). The strain showed the same features to *Issatchenkia orientalis* (Kurtzman, 1998) in the morphological characteristics and fermentation of various sugars including glucose, galactose, sucrose, maltose, lactose, and raffinose as well as in the growth in the presence of 10% NaCl in a media containing 5% glucose. Growth at 37 or 40°C was the same with each other. Assimilation patterns of the 23 out of 26 carbon sources tested in this study were also the same with each other. However, it showed different characteristics in the assimilation of various carbon sources. The strain KMBL 5774 could assimilate several carbon sources such as inulin, D-xylose and D-ribose, which is different features from *Issatchenkia orientalis* described by Kurtzman previously (1998).

ITS (internal transcribed spacer) sequencing and phylogenetic analysis
The yeast ITS I-5.8S rDNA-ITS II sequences have been known to be variable among the yeast strains (Granchi *et al.*, 1999; Park *et al.*, 1999; Torija *et al.*, 2001). Because there were some differences in the assimilation patterns between the isolated strain KMBL 5774 and *Issatchenkia orientalis* described by Kurtzman previously (1998), we have also determined the nucleotide sequences of ITS I-5.8S rDNA-ITS II region strains to confirm the identification of the strain KMBL 5774. About 600 bp DNA fragment containing ITS I-5.8S rDNA-ITS II region was amplified from the chromosomal DNA of the isolate and its sequences were determined. Phylogenetic analysis of the 420 nucleotide sequences of ITS I-5.8S rDNA-ITS II region of the strain KMBL 5774

Table 1. Morphological and physiological characteristics of the isolate KMBL 5774

Characteristics	KMBL 5774	*Issatchenkia orientalis*
Morphological characteristics		
Form	Elogate	Ovoidal to elogate
Vegetative reproduction	Budding	Budding
Spore formation	Ascosporogenous	Ascosporogenous
Pseudomycelium	Absent	Absent
True mycelium	Absent	Absent
Fermentation		
Glucose	+	+
Galactose	-	-
Sucrose	-	-
Maltose	-	-
Lactose	-	-
Raffinose	-	-
Assimilation		
Glucose	+	+
Galactose	-	-
L-Sorbose	-	-
Sucrose	-	-
Maltose	-	-
Lactose	-	-
Raffinose	-	-
Inulin	+	-
Soluble starch	-	-
D-Xylose	+	-
D- or L-Arabinose	-	-
D-Ribose	+	-
L-Rhamnose	-	-
Methanol	-	-
Ethanol	+	+
Glycerol	+	+
Erythritol	-	-
Galacitol	-	-
D-Mannitol	-	-
D-Glucitol	-	-
Salicin	-	-
D-, L-Lactate	+	+
Succinate	+	+
Citrate	w	+/w
Inositol	-	-
Nitrate	-	-
Others		
Growth at 10% NaCl/5% glucose	+	+
Growth at 37°C	+	+
Growth at 40°C	+	+

showed that it is closely related to *I. orientalis* zhuan 192 and several other strains of *Issatchenkia* sp. (Fig. 1). When the sequences of the strain KMBL 5774 and *I. orientalis* zhuan 192 was aligned, there were only a few differences in their nucleotide sequences. Identity of the sequences of the strain KMBL 5774 with those of the strain zhuan 192 was 99.5%. The sequences containing ITS I region and 5.8S rDNA of the strain KMBL 5774 were the same as those of the strain zhuan 192. Only 1 nucleotide deletion and 1 nucleotide insertion in the ITS II region of the strain KMBL 5774 were found compared with those of the strain zhuan 192 (data not shown). Therefore, the isolated strain KMBL 5774 was identified as a strain of *I. orientalis*.

Degradation of malic acid in YNB-malic acid media by I. orientalis KMBL 5774

Degradation of malic acid by *I. orientalis* KMBL 5774 was investigated in YNB-malic acid liquid media under various culture conditions such as malic acid concentration, pH, temperature, sorbitol, nitrogen source, etc. When the effects of the initial malic acid concentration in a range of 0 to 8% were investigated, the maximal growth (O.D. 10.7 at 600 nm) was obtained when 3% malic acid was used as a carbon source. In the presence of 2 or 4% malic acid, almost same level of cell growth was observed. The cell growth was inhibited when over 5% malic acid was used. Maximal malic acid degradation ratio was obtained at 1 or 2% of malic acid concentration, which were 94.4 and 94.6%, respectively. However, the optimal malic acid concentration for the growth was 3%, in which the malic acid degradation was 92.3%. When 8% malic acid was used, cell growth decreased and reached only O.D. 4.1 at 600 nm and about 75.2% of the malic acid was degraded (Fig. 2).

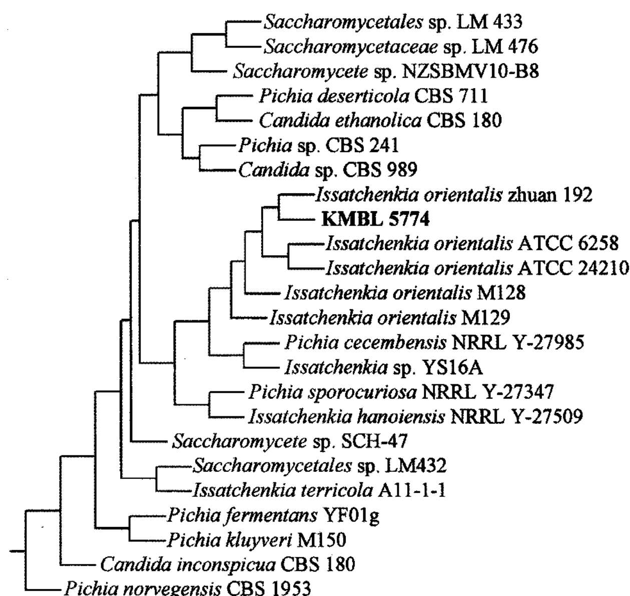

Fig. 1. Phylogenetic tree based on the ITS I-5.8S rDNA-ITS II sequences of *I. orientalis* KMBL 5774 and other related yeasts. The tree was constructed by using the neighbor-joining method and the Kimura two-parameter calculation model (Kimura, 1980).

When the effects of the initial pH were investigated, the yeast cells could grow in an acidic pH range from 2 to 4. The maximal growth (O.D. 10.6 at 600 nm) and malic acid degradation (95.5%) were obtained at pH 3.0 and 2.0, respectively. The growth at pH 2.0 was about 9.9 at 600 nm, which is about 93.4% of that at pH 3.0. The yeast showed O.D. 9.5 at 600 nm at pH 4.0. These results suggest that the isolated strain is an acidophilic yeast. According to the increase of the initial pH of the media in a range of pH 4.0 to 8.0, its cell growth at 600 nm decreased together

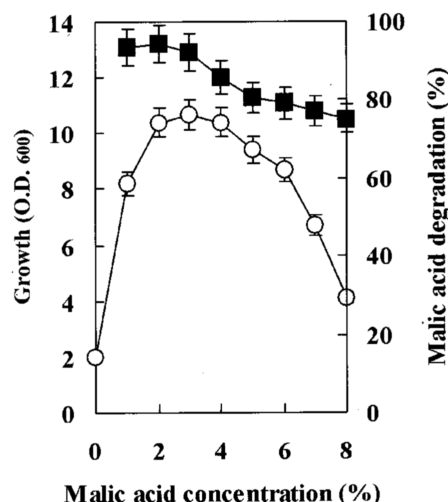

Fig. 2. Effects of malic acid concentrations on the degradation of malic acid by *I. orientalis* KMBL 5774. The yeast cells were grown at 30°C for 24 h with shaking (150 rpm) in YNB media (pH 2.0) containing various concentrations of malic acid as the sole carbon and energy source. After the culture, cell growth at 600 nm (○) and malic acid degradation ratio (■) were assayed.

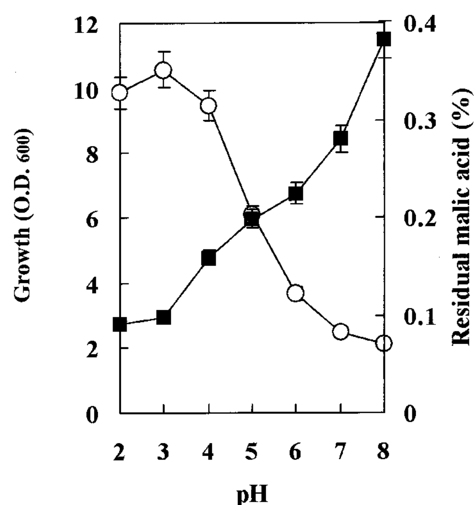

Fig. 3. Effects of initial pH on the degradation of malic acid by *I. orientalis* KMBL 5774. The yeast cells were grown at 30°C for 24 h with shaking (150 rpm) in YNB-2% malic acid media whose pH were adjusted to the range shown below the figure. After the culture, cell growth at 600 nm (○) and malic acid degradation ratio (■) were assayed.

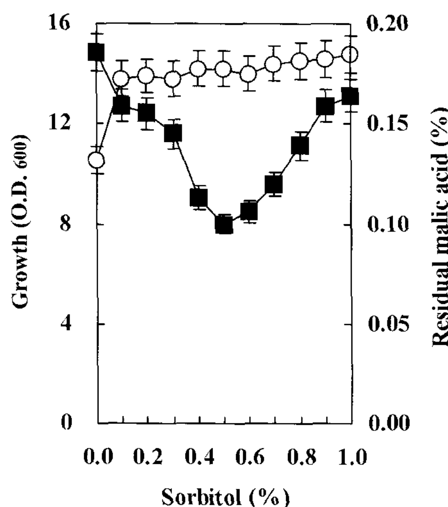

Fig. 4. Effects of sorbitol on the degradation of malic acid by *I. orientalis* KMBL 5774. The yeast cells were grown at 30°C for 24 h with shaking (150 rpm) in YNB-2% malic acid media (pH 2.0) containing various concentrations of sorbitol. After the culture, cell growth at 600 nm (○) and malic acid degradation ratio (■) were assayed.

with the increase of the residual malic acid content. It is interesting to notice that the yeast degraded about 81.0% of the malic acid added in the media at pH 8.0 though its cell growth reached only 2.1 at 600 nm. This might be due to the fact that the yeasts could not propagate very well at a high pH but could still transport the malic acid in the media into the cell (Fig. 3).

When effects of various carbon sources on the malic acid degradation by the isolate was tested, it was found that the addition of 0.2% sorbitol increased the cell growth of *I. orientalis* KMBL 5774 and malic acid degradation in YNB-2% malic acid media (data not shown). Therefore, we have tested the effects of sorbitol on the malic acid degradation more carefully. Without sorbitol, the residual malic acid content was 0.186% after the culture of the isolate at 30°C for 24 h. When sorbitol was added at a concentration less than 0.5% in the media, the residual malic acid decreased with the increase of the cell growth. Maximal degradation of malic acid was observed and the residual malic acid content was lowered to 0.1% when 0.5% sorbitol was added in the media. However, more amount of sorbitol than 0.5% was added in the media, the residual malic acid content rather increased after the culture (Fig. 4).

Discussion

This is the first report that *Issatchenkia orientalis* is an acidophilic yeast strain, which could degrade malic acid very efficiently. *I. orientalis* has been isolated as an ethanol using yeast with acid and ethanol tolerance (Okuma *et al.*, 1986) and as one of the indigenous yeasts present in the wine for the first time in 2004 (Clemente-Jimenez *et al.*, 2004). However, its beneficial function in the wine fermentation has not been studied such as malic acid degradation thus far.

A yeast strain, designated as KMBL 5774, degrading malic acid as the sole carbon and energy sources was isolated from Korean grape wine pomace. The strain was identified as *Issatchenkia orientalis* based on its morphological and physiological characteristics by the method of Kurtzman (1998) (Table 1). It is confirmed by a molecular biology method as well. Recently, molecular biology methods have been applied to the identification of yeasts. DNA-based methods have the advantage of being independent of gene expression (Ness *et al.*, 1993). In this sense, methods based on polymerase chain reaction (PCR) have been shown to be the most appropriate tools for rapid yeast identification. The differences in the nucleotide sequences of the internal transcribed spacer (ITS) I-5.8S rDNA-ITS II region have been used to identify yeast species during spontaneous fermentation of natural juices such as grape (Granchi *et al.*, 1999; Torija *et al.*, 2001) or orange (Las Heras-Viazquez *et al.*, 2003). Therefore, the DNA region containing the ITS I-5.8S rDNA-ITS II sequences were amplified by PCR and its sequences were analyzed to confirm the identification of the strain. Phylogenetic analysis showed the isolate is the closest to the *I. orientalis* zhuan 192 (Fig. 1). Alignment of their sequences showed that the identity of the sequences of the KMBL 5774 with those of zhuan 192 was 99.5%. Only 1 nucleotide deletion and insertion in the ITS II region were found in the KMBL 5774 compared with those of zhuan 192.

The isolate could degrade malic acid rapidly in the media containing 2% malic acid whose pH is 2.0 in the presence of 0.5% sorbitol (Fig. 3 and 4). There have been a large number of researches on the isolation and characterization of microorganisms, especially yeast strains, degrading malic acid. Because it has been well known that malic acid is sometimes detrimental to the quality of wines because of its too much contents in some varieties (Volschenk *et al.*, 1997, 2001). In addition, several grape varieties, especially which were grown in the cooler regions, have been reported to contain considerably high amount of malic acid enough to affect negatively the quality of wine (Ruffner, 1982). However, *Saccharomyces* sp., the major species used for the wine brewing cannot degrade malic acid efficiently. Typically, strains of *Saccharomyces* sp. are regarded as inefficient metabolizers of extracellular malic acid. Moreover, some strains of *Saccharomyces* sp. have been reported to synthesize of malic acid (Fatichenti *et al.*, 1984; Schwartz and Radler, 1988; Pines *et al.*, 1996, 1997; Ramon-Portugal *et al.*, 1999). In general, the ability to metabolize extracellular malic acid depends on an efficient uptake system for malic acid (i.e. active import via a malate transporter) and a malic acid-converting enzyme (i.e. fumarase, malolactic enzyme, malate dehydrogenase or a malic enzyme). In contrast, strains of *Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* can degrade high concentrations of malic acid (Kuczynski and Radler, 1982; Baranowski and Radler, 1984; Osothsilp and Subden, 1986; Osothsilp, 1987; Taillandier *et al.*, 1988; Rodriquez and Thornton, 1989; Taillandier and Strehaiano, 1991).

I. orientalis KMBL 5774 isolated in this study could degrade malic acid regardless of the presence of assimilatable sugars in the media suggesting that the strain belongs to K(+) group (Fig. 3 and 4) although the addition of 0.5%

sorbitol could increase the cell growth and malic acid degradation (Fig. 5). Yeast species degrading malic acid can divided into two groups, either K(-) or K(+) yeast group, depending on their ability to utilize L-malic acid and other tricarboxylic acid (TCA) cycle intermediates as sole carbon and energy sources (Saayman *et al.*, 2000). Genetic and biochemical characterization of the malic acid-utilizing pathways in several K(-) and K(+) yeast species, including *S. pombe, Candida utilis, Kluyveromyces marxianus, Z. bailii*, and *Saccharomyces cerevisiae*, indicated that the physiological role and regulation of malic acid metabolism differs significantly between the K(-) and K(+) yeasts. The K(+) group includes *Candida sphaerica* (Corte-Real *et al.*, 1989), *Candida utilis* (Cassio and Leao, 1993), *H. anomala* (Corte-Real and Leao, 1990), *Pichia anomala* (Amador *et al.*, 1996) and *K. marxianus* (Queiros *et al.*, 1998), which have the ability to utilize TCA cycle intermediates as sole energy and carbon sources, with no requirement for other assimilatable sugars. The K(-) group can utilize TCA cycle intermediates only in the presence of glucose or other assimilable carbon sources (Barnett and Kornberg, 1960). Strains of *Saccharomyces* sensu stricto (*S. cerevisiae, C. paradoxus, S. pastorianus, S. uvarum, S. bayanus*), *S. pombe* and *Z. bailii* are all classified as K(-) yeasts. Although grouped together, the yeasts in this category have diverse aptitudes to metabolize malic acid.

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