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Malo-lactic Bacteria in Korean Winery Environment and Their Potential Use in Wine Making

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한국내 양조 환경하의 malo-lactic 박테리아의 분포 및 그들의 양조업에의 이용성

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Abstract

Substantial numbers of malo-lactic bacteria were detected in Korean winery environment such as in grape juices, fermenting musts, lees, aging wines, and bottled apple wines. Among 1363 malo-lactic strains isolated from the above habitats, four superior strains were selected and identified as *Leuconostoc oenos* strain A-25, B-30, C-13 and *Lactobacillus plantarum* strain D-11. The strain A-25 showed malate utilizing activity almost equivalent to *Leu. oenos* ML34, a well-known malo-lactic bacterium. Upon experimental vinifications the malo-lactic fermentation efficiency of A-25 was confirmed.

Introduction

Moderate acid test is desirable flavor characteristic of wine. The acid taste of wine comes from acid components, of grapes, mainly tartaric acid and malic acid. The amount of these acids in grapes varies depending on the variety, region and season. Consequently, the acidity of wine varies with the quality of grapes used. Another factor which affects the acid taste of wine is the conversion of malic acid (a dicarboxylic acid which may constitute as much as half of the acid of grapes) to lactic acid, a monocarboxylic acid. This conversion, now referred to as malo-lactic fermentation, results in a deacidification which often occurs during storage of new wine. Malo-lactic fermentation, therefore, may be desirable in wines of high acidity but is deteriorative to wines with acidities that are already low. Certain lactic acid bacteria are known to be involved in this conversion. Accordingly, the acidity of wine may be controlled by regulating these malo-lactic bacteria in the wine during storage as well as by selecting the type of grapes.

Malo-lactic fermentation may occur spontaneously during vinification but it is difficult to stimulate it consistently (4, 7, 12, 13, 14, 15, 19). Instead of relying on the natural microflora the use of pure culture inoculation with selected strains to stimulate the malo-lactic fermentation has been studied ext-

ensively (4, 5, 9, 12, 13, 14, 15, 20). The organism which receive the most attention was *Leuconostoc oenos* ML-34, a strain originally isolated by Ingraham et al (10) from a red wine made in California.

The average grapes grown in Korea, mostly of edible-type varieties, have relatively high acidities (17). Malo-lactic fermentation, therefore, may be needed either by natural microflora or by pure culture inoculation. In the present study the natural distribution of malo-lactic bacteria in Korean winery environment was surveyed to see the possibility of spontaneous conversion of malic acid to lactic acid during vinification. Isolation of powerful strains was also attempted for purpose of future use as pure culture inoculum to induce malo-lactic fermentation.

Materials and Methods

Isolation and selection of malo-lactic bacteria

The malate-utilizing bacteria were isolated from grape juices, fermenting musts, lees, and wines of a Korean domestic winery. Bottled grape wines of other domestic plants as well as apple wines were also collected from retail stores and used as bacterial sources. Tomato Juice Glucose Broth (T-GB, pH 4.5) which was originally developed by Pilone et al (18) was enriched with 0.3% L-malate and used to make pour plates of the samples in it solidified with 2% agar after treating with 50 mg/*l* Actidione (Cycloheximide, Sigma Chemical Co., Saint Louis, Missouri) to inhibit yeast growth. When each plate was hardened, another layer of agar was added on the surface. The finished plates were incubated at 25° C in desicators where the oxygen tension was reduced by using lighted candles. After colony counts (total facultative anaerobes) were made, the large colonies, which were considered to be malate-utilizing bacteria, were transferred separately into culture tubes having the same malate enriched TGB (pH 4.5) without agar and Actidione. The tubes were incubated at 25°C for 7 days with daily checkings for malate disappearance by means of paper chromatography (11).

Identification of selected malo-lactic bacteria

Four strains isolated from the lees after fermentation which showed strong malate-utilizing activity were selected and identified through the procedures described by Pilone et al (18) as well as referring to the Bergey's Manual (6). A well known malo-lactic species *Leuconostoc oenos* ML-34, a gift from Dr. G. J. Pilone, The Christian Brothers, Mont La Salle Vineyards, Napa, California, was also recharacterized along with the new isolates for the purpose of comparison.

Preparation of bacterial inocula

The selected malo-lactic strains were cultured in modified Rogosa medium (18) supplemented with 0.5% L-malic acid for malate-adaptation. After a week of incubation at 25°C, cells were harvested by centrifugation (10,000×g, 20 min), washed once with distilled water, and resuspended into distilled water to obtain a cell suspension of about 3×10^9 cells per ml. In order to adaptate the cells to grape juice, the cell suspension was inoculated at 1% level (v/v) in a medium containing 60 % grape juice, 0.2% L-malic acid, 0.05% $(NH_4)_3PO_4$, 0.0003% $MnSO_4.4H_2O$, and 50 ppm SO_2. The pH of the medium was adjusted to 4.5 with 6N HCl or KOH. After one week incubation the culture was used as bacterial inoculum to induce malate utilization during fermentation.

Vinification

Grapes of two varieties, Muscat Bailey A and Pinot noir, were used. The crushed juices were ameliorated with sucrose to 24 brix and filled in 20-liter fermentors. After addition of 65 ppm SO_2 the juices was inoculated with 2% (v/v) *Saccharomyces cerevisiae* var. *ellipsoideus* Montra yeast (UCD Enology #522) which was donated by Dr. R. E. Kunkee, University of California, Davis. When needed, the prepared bacterial inocula were applied at 1% level simultaneously. After Brix dropped nearly to zero, wines were racked, filtered using a filter aid (Hyflo Super Cell) and subjected to the analyses.

Chemical analyses

Malo-lactic fermentation was monitored by observing malate-disapperance on the paper chromatogram (11). Malic acid concentration was determ-

ined quantitatively (16) by enzymatic reduction of nicotin-amide adenine dinucleotide (Nutritional Biochemicals Corp.) with malic dehydrogenase (Sigma Co.). Total lactic acid was determined by the method of Barker and Summerson (3) and reducing sugar by Lane and Eynon procedure (2). Other analyses were made following methods described by Amerine et al (1).

Results and Discussion

Occurrence of malo-lactic bacteria in Korean winery environment

One of the Korean domestic wineries was surveyed stepwise following the production line to disclose the natural distribution of malo-lactic bacteria (Table 1). Apparently a substantial number of malo-lactic bacteria had been brought in the winery through fresh grapes. An average of 32 malo-lactic bacteria per ml of grape juice which occupied one fifth of total facultative anaerobic microflora was recorded. The number of malo-lactic bacteria increased during fermentation, but the rate against the number of total facultatives dropped to 4% and remained around the same level thereafter. Obviously, the environmental conditions within the fermentors and aging tanks were more favorable for other facultative anaerobes than the malo-lactic bacteria. Furthermore, in the bottled wine including samples of other wineries, no facultative anaerobes was present. This may due to the pasteurization and further addition of SO_2 (100ppm) which are normal procedures in Korean wineries before bottling. In the 15 bottles of domestic apple wines collected randomely from several retail stores showed some facultative anaerobes and half of which were of malo-lactic type. The high content of malic

Table 1. Distribution of malo-lactic bacteria in Korean wines and related materials.

Habitat		No. of samples	Average total facultative anaerobes/ml	Average malo-lactic bacterial/ml	Ratio* (%)
From a Korean Winery	Grape juice, fresh	13	1.5×10^2	32	21
	Fermenting must	14	2.3×10^3	91	4
	Lees after fermentation	16	4.6×10^3	236	5
	Grape wine in agiog tanks	14	2.8×10^3	84	3
	Grape wine, bottled	10	0	9	0
Frem other Korean winergy	Grape wine, bottled	6	0	0	0
	Apple wine, bottled	15	2.6	1.2	46

* $\frac{\text{malo-lactics}}{\text{total facultatives}}\times100=\%$

Table 2. Malo-lactic bacteria isolated from Korean wines and related materials and their malate utilizing activities

Habitat*	Total malo-lactic bacteria isolated	malo-lactic bacteria utilized malate completely in		
		5 days	7 days	7 days
Grape juice, fresh	83	0	6	77
Fermenting must	254	1	7	246
Lees after fermentation	755	4	19	732
Grape wine in aging tanks	235	0	6	230
Apple wine, bottled	36	0	5	31
Total	1,363	5	42	1,316

*Habitat and no. of sampling were the same as Table 1.

Table 3. Characterstics of selected malo-lactic strains

Genus species strain		Leuconostoc oenos ML 34	Leuconostoc oenos A-25	Leuconostoc oenos B-30	Leuconostoc oenos C-13	Lactobacillus plantarum D-11
Morphology		coccus	singly	in pairs and chains		rod, singly, in pairs, short chain
Type of fermentation		Hetero	Hetero	Hetero	Hetero	Homo
Gram stain		+	+	+	+	+
Presence of catalase		−	−	−	−	−
Gas production from glucose		+	+	+	+	−
Lactic acid isomer from glucose		D	D	D	D	DL
Dissimilation of (in presence of glucose)						
	Malic acid	+	+	+	+	+
	Citric acid	+	+	+	+	+
	Fumaric acid	−	+	+	+	+
Growth on	D-glucose	+	+	+	+	+
	D-fructose	+	+	+	+	+
	Galactose	−	−	−	−	+
	Raffinose	−	−	−	−	+
	Sucrose	−	−	−	−	+
	Lactose	−	−	−	−	+
	Maltose	−	−	−	−	+
	D-ribose	+	+	+	+	+
	D-xylose	−	−	−	−	+
	L-arabinose	−	−	±	±	±
	D-mannose	−	−	−	−	+
	L-sorbitol	−	−	−	−	+
	D-mannitol	−	−	−	−	+
	Esculin	+	+	+	+	+
	Salcin	+	+	+	+	+
Growth at	10°C	−	−	−	−	+
	25°C	+	+	+	+	+
	35°C	−	−	+	+	+
	45°C	−	−	−	−	−

+: positive response, −: negative response, ±: variable response

acid in apple juice might have provided better environment for the malo-lactic bacteria compared to the grape wine.

For the induction of malo-lactic fermentation a pure culture of bacteria is usually inoculated in the young wine before aging. One% (v/v) inoculum with 1×10^7 cells per ml culture will result in malo-lactic bacteria concentration of 1×10^5cell per ml of young wine. This is much higher than 84 malo-lactic bacteria per ml of wine in aging tank without inoculation (Table 1). Therefore, it may be concluded that, in Korea, a sufficient reduction of malic acid in aging wine can not be expected in the spontaneous malo-lactic fermentation. This may be specially true in wines made of grapes such as Muscat Bailey A, Delaware, and Golden Queen which were reported to be high in acidity (17).

Isolation and identification of potent malo-lactic bacteria

From the seven different habitats totally 1363-malo-lactic strains were isolated and categolized

into three groups based on their malate utilizing abilities such as those which utilize the 0.3% malate in the medium completely in 5 days, 7 days, and more than 7 days (Table 2). Among them only five strains could utilize malate completely under the culture conditions and four of which from the lees after fermentation were subjected to identification procedures.

As shown in Table 3 the characteristics of the three newly isolated strains, i.e., strain A-25, B-30, and C-13 were identical with the model species of *Leuconostoc oenos* ML 34. The last isolate, D-11, agreed nicely to the descriptions for *Lactobacillus plantarum* appeared in Bergey's Manual(6). This species has also known to be a malo-lactic organism (8).

Table 4. Time required for complete removal of malic acid in wine* during fermentation.

Inoculum	Duration of malo-lactic fermentation (weeks*)
Yeast**	>25.0
Yeast+*Leu. oenos* ML34	13.8
Yeast+*Leu. oenos* A-25	14.5
Yeast+*Leu. oenos* B-30	15.5
Yeast+*Leu. oenos* C-13	18.2
Yeast+*Lac. plantarum* D-11	25.0

*Muscat Baily A grape was used.
***Saccharomyces cerevisiae* var. *ellipsoideus*
***Time to disapear malate spot with simultaneous appearance of lactate spot on paper chromatogram

Malo-lactic fermentation of isolated bacteria

The newly isolated four strains of bacteria were inoculated separately in grape juice along with regular wine yeast and their malo-lactic fermentation abilities were examined by determining the fermentation time to reach a complete removal of malate in the wine. For the purpose of comparison the well-known malo-lactic strain *Leu. oneos* ML 34 was also tried. As can be seen in Table 4 a wide spectrum of ability was detected within the strains tested. Among the newly isolated strains *Leu. oenos* A-25 showed the highest malo-lactic fermentation ability which was very close to that of ML 34.

Table 5 shows the malo-lactic fermentation efficiency of the strain A-25 in wine fermentation more clearly. The high malate content in the raw material (must) could be removed almost completely with the concomitant increase of lactate content in the wine fermented with A-25 (MLF) whereas the wine without this (control) showed little change. Substantial changes in total acidity and pH value were also accompanied in the MLF wines. The trend was same in both wines made with different varieties of grapes.

요 약

한국내 양조장을 둘러싼 미생물 환경 즉 신선한 포도즙, 발효중의 포도즙, 앙금, 숙성중의 포도주 및 병속의 사과주들 속에서 많은 수의 malo-lactic 박테리아가 검출되었다. 이들의 환경 속에서 분리된 1363 주의 malo-lactic 균주들 가운데 우수한 4균주를 선택하여 분류한 결과 *Leuconostoc oenos*

Table 5. Malo-lactic function of newly isolated *Leuconostoc oenos*A-25 in wine fermentation

Grape used	Product	Malic acid (mg/100ml)	Lactic acid (mg/100ml)	Total acidity (g tartrate/100ml)	pH
Muscat Baily A	Must	360	0	1.07	3.22
	Wine, control*	351	trace	1.07	3.26
	Wine, MLF**	trace	158	0.68	3.52
Pinot noir	Must	358	0	1.02	3.20
	Wine, control	336	trace	0.98	3.24
	Wine, MLF	trace	175	0.63	3.54

*Wine fermented with yeast (*S. cerevisiae* var. *ellipsoideus*) alone.
**Wine fermented with yeast+*Leu. oenos* A-25.

종에 속하는 3균주인 A-35, B-30, C-13과 *Lactobacillus plantarum*종에 속하는 1균주 D-11을 얻었다. 이들 가운데 A-25균주는 malo-lactic 박테리아로서 널리 이용되고 있는 *Leu. oenos* ML34와 거의 동등한 사과산 이용능력을 보였다. 실험적인 양조를 통하여 이 A-25균주의 malo-lactic 효과를 확인 하였다.

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