EFFECTS OF MALO-LACTIC FERMENTATIVE BACTERIA ON THE ACIDITY OF WHITE WINE[1]

HENG-CHAO LIU[2] and SAN-SAN TSAY LIU[3]

(Received for publication January 1981)

Abstract: Three malo-lactic species of lactic acid bacteria were isolated from grape juice and fermented dairy products and identified as *Pediococcus cerevisiae*, *Streptococcus lactis* subsp. *diacetylacetis* and *Pediococcus pentosaceus*. Malo-lactic fermentation was induced in separate lots of wine by inoculation of each lot with one of the isolated bacteria. Yeast extract could be added to improved the nutritional content of the wine. Malo-lactic fermentation had occurred in each inoculated wine within 42 days. The resultant wines were subjected to chemical analyses. A measurable decrease in fixed acidity was observed, but no significant change in volatile acidity was noted in any of the wines. When the fermentation was complete, a stoichiometric relation was found between malic acid lost and lactic acid produced.

INTRODUCTION

The malo-lactic fermentation (the conversion of malic acid to lactic acid and CO_2), usually occurring after the alcoholic fermentation of grape juice, is caused by certain lactic acid bacteria. This reaction has been noted in all the wine-producing areas of the world[18], including Europe, Australia[26], South Africa, and the United States[11,17,27,32].

Deacidification of wines has been known since the beginning of the Science of Microbiology. However, the earlier workers (Pasteur, Berthelot and de Fleurieu) were primarily interested in the decomposition of tartrates. Until 1891, the fermentation of malic acid in wine was seriously attended to by the investigation of Muller-Thurgau. Muller-Thurgau and his contemporaries (Kock, Moslinger, Seifert) found that bacteria, not yeast, were responsible for the decomposition of malic acid with the formation of lactic acid and CO_2 as major end products, and caused a reduction in total acidity of wines[12,18]. Thus, the malo-lactic fermentation is very important in the wine with high acidity. The purpose of this paper is to study the effects of malo-lactic fermentative bacteria on the acidity of Taiwan local wine.

MATERIALS AND METHODS

Bacteria

The malo-lactic bacteria were isolated from grape juice and fermented milk product, yogurt. The medium used for the isolation are modified Rugosa medium according to Chalfan *et al.*[6,18]. We followed the method described by Pilone and Kunkee[22,24] to isolate malo-lactic bacteria. At last, the bacteria were examed by standard methods given in Laboratory Excer-

(1) This paper is based partly on a M.S. thesis of the first author to Research Institute of Botany, NTU and was supported by a research grant from Wine Research Institute, Taiwan Tobacco and Wine Monopoly Bureau.

(2) 劉恒昭, Teaching Assistant, Botany Department, NTU.

(3) 蔡珊珊, Associate Professor, Botany Department NTU.

cises in Microbiology[20] and classified according to Bergey's Manual of Determinative Bacteriology[5].

Wine sample

The sample of white wine was obtained from Nantau Winery produced in July 1979. The wine, sterilized at 65°C for 20 min, has a pH value of 3.15. The pH of wine is adjusted with 10N NaOH to pH 4.10. In some experiments, 2×10^{-4} g/ml yeast extract was added[21].

Chemical Analyses

The wine was analyzed for pH, total acidity and volatile acidity according to the methods given by Amerine[3], and AOAC[4]. Organic acids in wine was separated by ion exchange method given by Tarantola and Castino[1,2]. Malic acid was determined qualitatively by paper chromatography technique of Kunkee[3,24], and determined quantitatively by the modified method of Tarantola *et al.*[2]. Lactic acid concentration was determined quantitatively by the colorimetric procedure of Pilone and Kunkee[3,23].

Preparation of Bacterial inocula

Bacterial cultures used for inoculation of wine were prepared by the procedure of Kunkee, Ough and Amerine[16]. In the present paper, 1% bacterial inoculum indicates the addition an equivalent of 1 volume of culture, before centrifugation, to 100 volumes of wine. Bacterial concentration in wine is determined by plate-counting method using tomato juice agar plate at 25°C for 2–3 days.

RESULTS

Bacterial identification

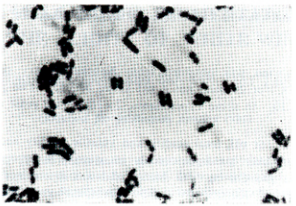
Three species of malo-lactic bacteria were isolated. By definition, malo-lactic bacteria are lactic acid bacteria which can ferment malic acid. They are members of the families of *Lactobacillaceae* and *Streptococcaceae*, the former is rod and the latter is coccus. Since the isolates in this experiment are Gram-positive, catalase-negative, facultative anaerobic, and lactic acid producing bacteria with coccus in cell morphology, they all belong to the Family of *Streptococcaceae*. Isolates CA-3 and FE-4 were classified to the genus of *Pediococcus* since they are homofermentatitive with tetrad in cell arrangement, while isolate FA-3 was classified to the genus *Streptococcus* as it is homofermentative but not tetrad in cell arrangement. Further division of the three isolates is dependent on the optimal temperature of growth, kinds of sugars fermented and other characteristics given in Bergey's Manual of Determinative Bacteriology[5,7,10,33]. They were identified as *Pediococcus cerevisiae*, *Streptococcus lactis* subsp. *acetylactis* and *Pediococcus pentosaceus* (Fig. 1).

pH and total acidity

The malo-lactic fermentation of isolated bacteria was completed in 6 weeks after the initial bacteria inoculation. Bacteriological and chemical analyses were made on the weekly samples from each lot of wine. The pH changes in the wine of *P. cerevisiae* are presented in Table 1. In order to observe the changes of pH and acidity, the amount of inoculum must be over 15%. Significant decrease in the acidity was observed at higher temperature and higher amount of inoculum. For the decreasing of the total acidity, the addition of yeast extract was found to be more efficient than the change of temperature. Total acidity can be reduced to a maximum value of 0.15 g/100 ml (tartaric acid), compared to the uninoculated wine (Fig. 2) of *P. cerevisiae*. The other two isolated showed similar results in changes of pH and acidity.

Volatile acidity

The volatile acidity, given in Table 2, remained quite constant during growth of the malo-



(a)

(b)

(c)

Fig. 1. Photomicrograph of (a) *Pediococcus cerevisiae*, (b) *Streptococcus lactis* subsp. *diacetylactis*, (c) *Pediococcus pentosaceus*. ×1350

Table 1. pH value at the 42nd days of malo-lactic fermentation by *Pediococcus cerevisiae*

pH of original wine	Temp.	Bacterial inocula percentage and YE supplement*									
		100%		75%		50%		25%		15%	
		+YE	−YE	+YE	−YE	+YE	−YE	+YE	−YE	+YE	−YE
3.15	25°C	3.28	3.26	3.26	32.2	3.24	3.22	3.20	3.18	3.16	3.16
	15°C	3.26	3.24	3.22	—	3.20	—	3.18	—	—	—
4.10	25°C	4.24	4.22	4.22	4.20	4.20	4.20	4.18	4.16	4.20	4.15
	15°C	4.24	4.22	4.22	4.22	4.20	4.20	—	—	—	—

* YE: Yeast extract
conc. of YE supplement: 2×10^{-4} g/ml

lactic organism. The amount of inoculum, change of temperature and addition of yeast extract have only very little effects. The volatile acidity could only be increased by a maximum value of 0.026 g/100 ml (acetic acid), compared to the uninoculated wine.

Malic and lactic acidity

Malic acid was separated from wine by ion exchange method, then determined qualitatively and quantitatively. The malic acid and lactic acid content in the wine of *P. cerevisiae* shown

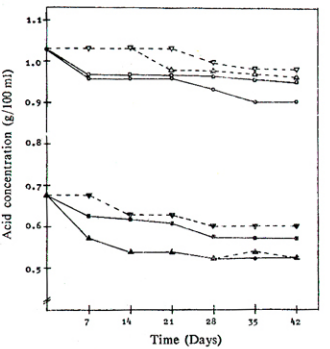


Fig. 2. Changes in total acid concentration of wine fermented by 100% inoculum of *Pediococcus cerevisiae*.

- •—• pH 4.10, 25°C, +YE
- ■—■ pH 4.10, 25°C, −YE
- ▲···▲ pH 4.10, 15°C, +YE
- ▼···▼ pH 4.10, 15°C, −YE
- ○—○ pH 3.15, 25°C, +YE
- □—□ pH 3.15, 25°C, −YE
- △···△ pH 3.15, 15°C, +YE
- ▽···▽ pH 3.15, 15°C, −YE

Table 2. Volatile acidity in Malo-lactic fermented wine

Species	wine pH	Temp.	Volatile acidity as acetic acid (g/100 ml) 100% +YE	100% −YE	75% +YE	75% −YE	50% +YE	50% −YE	25% +YE	25% −YE	15% +YE	15% −YE	Original wine
Pediococcus cerevisiae	3.15	25°C	.037	.037	.037	.036	.037	.036	.036	.036	.034	.034	.034
		15°C	.037	.036	.036	—	.036	—	.035	—	—	—	.034
	4.10	25°C	.060	.041	.041	.041	.040	.039	.039	.038	.038	.037	.034
		15°C	.040	.038	.039	.038	.039	.039	—	—	—	—	.034
Streptococcs lactis subsp. *diacetylactis*	3.15	25°C	.038	.036	.037	.036	.037	.035	.035	.035	.035	.034	.034
		15°C	.036	.035	.035	—	.035	—	.035	—	—	—	.034
	4.10	25°C	0.41	.040	.040	.039	.038	.036	.037	.036	.034	.034	.034
		15°C	.039	.038	—	—	.036	.035	.035	.034	—	—	.034
Pediococcus pentosaceus	3.15	25°C	.037	.036	.036	.036	.036	.035	.035	.035	.034	.034	0.34
		15°C	.036	.036	.035	—	.036	—	.035	—	—	—	.034
	4.10	25°C	.041	.038	.040	.038	.038	.037	.038	.037	.036	.036	.034
		15°C	.040	.037	—	—	.038	.037	.037	.036	—	—	.034

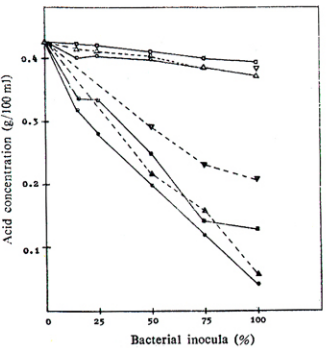


Fig. 3. Changes in malic acid concentration of wine fermented by *Pediococcus cerevisiae*.

•—• pH 4.10, 25°C, +YE
■—■ pH 4.10, 25°C, −YE
▲---▲ pH 4.10, 15°C, +YE
▼---▼ pH 4.10, 15°C, −YE
○—○ pH 3.15, 25°C, +YE
□—□ pH 3.15, 25°C, −YE
△---△ pH 3.15, 15°C, +YE
▽---▽ pH 3.15, 15°C, −YE

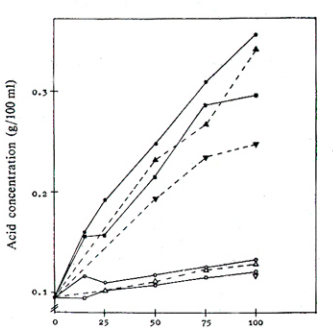


Fig. 4. Changes in lactic acid concentration of wine fermented by *Pediococcus cerevisiae*.

•—• pH 4.10, 25°C, +YE
■—■ pH 4.10, 25°C, −YE
▲---▲ pH 4.10, 15°C, +YE
▼---▼ pH 4.10, 15°C, −YE
○—○ pH 3.15, 25°C, +YE
□—□ pH 3.15, 25°C, −YE
△---△ pH 3.15, 15°C, +YE
▽---▽ pH 3.15, 15°C, −YE

in Figs. 3, 4 respectively, were strongly affected by the pH value of the wines, then by the addition of the yeast extract, and slightly affected by the change of temperature. The concentrations of malic acid in the uninoculated wine were 0.427 g/100 ml and 0.095 g/100 ml respectively. After malo-lactic fermentation, the malic acid content in the wine of pH 3.15 could decrease 0.06 g/100 ml while it could decrease as much as 0.39 g/100 ml in the wine of pH 4.10. The lactic acid content in the wine of pH 3.15 was increased less than 0.04 g/100 ml while it was increased more than 0.26 g/100 ml in the wine of pH 4.10. The other two isolated showed similar results in changes of malic and lactic acidity. Apparently, the wine with a higher pH value, 4.10, is better for the induction of malo-lactic fermentation.

Bacterial concentration

The changes of bacterial concentration in the wines after molo-lactic fermentation were also measured (Table 3). It was found that the bacterial concentration in the wines were strongly affected by the addition of yeast extract, then the change of temperature, and the pH value of the wines had only very little effects. It is obvious that the yeast extract can improve the nutritional content of the wine.

Table 3. Bacterial count in fermented wine of 100% inocula

Bacterial species	Original bacterial count (cell/ml)	pH of wine	YE supplement	Bacterial count (cells/ml)	
				25°C	15°C
Pediococcus cerevisiae	1.4×10^{8}	3.15	+	1.7×10^{9}	3.2×10^{7}
			−	7.0×10^{6}	1.2×10^{6}
		4.10	+	2.2×10^{8}	9.5×10^{7}
			−	7.3×10^{6}	1.8×10^{6}
Streptococcus liacts subsp. *diacetylactis*	1.1×10^{9}	3.15	+	6.7×10^{9}	7.3×10^{7}
			−	4.5×10^{7}	2.7×10^{7}
		4.10	+	8.2×10^{8}	3.3×10^{8}
			−	4.7×10^{7}	3.1×10^{7}
Pediococcus pentosaceus	1.0×10^{9}	3.15	+	5.2×10^{9}	1.6×10^{8}
			−	2.7×10^{7}	1.4×10^{7}
		4.10	+	7.6×10^{8}	4.0×10^{8}
			−	3.4×10^{7}	2.2×10^{7}

DISCUSSION

There are many difficulties when malo-lactic bacteria are inoculated to activate the malo-lactic fermentation in wine: (a) The antiseptic quality of alcohol is well known, therefore its presence in wine has an inhibitory effect on malo-lactic bacteria. The legal limit of alcohol in wine is about 10 to 14%, this concentration will delay the malo-lactic fermentation. (b) SO_2 is strongly inhibitory to malo-lactic bacteria, so the concentration in wine must be below 50 mg/l. (c) Wine is highly acidic and it has been reported that malo-lactic fermentation only occurs in wine with a pH value not below 3.0[28]. (d) Temperature could affect the metabolic pathways of the fastidious lactic acid bacteria. Malo-lactic fermentation is inhibited at 5° to

10°C, but it occurs at cellar temperature of 10°C to 15°C[18]. To solve these problems, we could increase the bacterial inoculum, titrate the wine with NaOH to pH 4.10, and add yeast extract to improve the nutrition of wine. Then, the container sealed tightly and incubated at 15° or 25°C[25,31].

Malo-lactic fermentation was completed in each inoculated wine within 42 days. Only a few different in composition were found when the malo-lactic wines were compared with one another. This result is the same as Pilone's report in 1965 and 1966[21,22]. During malo-lactic

Table 4. Stoichiometric relationship of the Malo-lactic fermentation. Bacterial inocula: 100%

Species	pH of wine	Temp.	YE supplement	Malic acid (g/100 ml)			Lactic acid (g/100%)				Difference between obs. & calc.
				before MLF	after MLF	loss by MLF	before MLF	aftre MLF	pro-duced by MLF	calc. from M. A.	
Pediococcus cerevisiae	3.15	25°C	+	.427	.372	.055	.095	.133	.038	.037	+.001
			−	.427	.391	.036	.095	.120	.025	.024	+.001
		15°C	+	.427	.377	.050	.095	.130	.035	.034	+.001
			−	.427	.396	.031	.095	.117	.022	.021	+.001
	4.10	25°C	+	.427	.041	.386	.095	.357	.262	.259	+.003
			−	.427	.127	.300	.095	.295	.200	.201	−.001
		15°C	+	.427	.056	.371	.095	.343	.248	.249	−.001
			−	.427	.202	.225	.095	.248	.153	.151	+.002
Streptococcus lactis subsp. *diacetylactis*	3.15	25°C	+	.427	.382	.045	.095	.128	.033	.030	+.003
			−	.427	.391	.036	.095	.117	.022	.024	−.002
		15°C	+	.427	.396	.031	.095	.117	.022	.021	+.001
			−	.427	.402	.025	.095	.110	.015	.017	−.002
	4.10	25°C	+	.427	.052	.375	.095	.243	.248	.252	−.008
			−	.427	.151	.276	.095	.277	.182	.184	−.002
		15°C	+	.427	.157	.270	.095	.278	.183	.181	+.002
			−	.427	.209	.218	.095	.233	.138	.146	−.008
Pediococcus pentosacus	3.15	25°C	+	.427	.367	.060	.095	.133	.038	.040	−.002
			−	.427	.414	.013	.095	.110	.015	.009	+.006
		15°C	+	.427	.385	.042	.095	.125	.030	.028	+.002
			−	.427	.411	.016	.095	.105	.010	.011	−.001
	4.10	25°C	+	.427	.016	.411	.095	.370	.275	.276	−.001
			−	.427	.101	.326	.095	.310	.215	.219	−.004
		15°C	+	.427	.057	.375	.095	.348	.253	.252	+.001
			−	.427	.166	.261	.095	.272	.177	.175	+.002

fermentation, total acidity decreased day by day, but volatile acidity remained relatively constant. The lactic acid found during the malic to lactic conversion bore a stoichiometric relationship to the malic acid lost, as calculated from the equation: $C_4H_6O_5$ (malic acid) $\longrightarrow$ $C_3H_6O_3$ (lactic acid)+CO_2. The differnces (Table 4) between the lactic acid found by analysis and that calculated from the amount of malic acid present were observed. This differences were not statistically significant. Therefore no lactic acid could be attributed to the metabolism of compounds other than malic acid by these bacteria during their growth cycle in the wine[25].

Rankine and Pilone[15] have found that the specific nutrition in wine needed for growth of the bacteria only presented in very low concentration, thus the complete growth of these inoculated bacteria was prevented. Yeast extract could be added to improve the nutritional content of the wine and then increase the concentration of bacteria (Table 3).

According to the experimental results, we could find that the malo-lactic fermentation was strongly affected by the pH value of the wine, then by the addition of the yeast extract, and slightly affected by the change of temperature. Since the malo-lactice fermentation is induced by malic enzyme and lactic dehydrogenase produced by malo-lactic bacteria[11,12,13,14,19], the activity of these enzymes are inhibited by low pH value, therefore the efficiency of malo-lactic fermentation could not raise with the increase of bacterial concentration.

Malo-lactic fermentation is necessary not to deacidify some wines but also to improve some wines apparently by addition of certain metabolic products which make the flavor more complex[23]. In this experiment, we had studied the deacidification of the wines, but more informations about the change of the composition in malo-lactic wines such as the concentration of alcohol, reducing sugars[3], proteins, vitamins and flavor products, etc. can be found by further studies.

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