[J. Ferment. Technol., Vol. 59, No. 1, p. 27–32, 1981]

Effects of Yeast Strains and Environmental Conditions on Formation of Organic Acids in Must during Fermentation*

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The effects of yeast strains and environmental conditions on the formation of organic acids during fermentation were examined with pasteurized Koshu grape musts. Twenty strains of yeast influenced considerably the formation of succinic and acetic acids by fermentation. Succinic acid was formed in quantity by *Saccharomyces cerevisiae* IAM 4274 and *Sacch. bailii* NISL TK-1, and acetic acid by *Candida krusei* IAM 4801. *Hansenula anomala* IAM 4721 produced much citric acid in addition to acetic acid. Formation of succinic and acetic acids was affected by fermentation temperature, sugar concentration, and pH of the must and the yeast strain used, while lactic and citric acid levels in the finished wines were not influenced by these factors.

A selected pure culture of yeast is commonly used for favorable fermentation of musts in wine making. However, in some small wineries it is customary to allow the must to ferment spontaneously without adding a starter yeast.[1-4] Dittrich[5] reported that wines fermented by pure cultures differed in total acids, glycerol content and volatile components from those fermented spontaneously. This suggests that the types of organic acid produced by wine yeasts depend greatly on the yeast flora in the must and the environmental conditions during fermentation. The fermentative formation of succinic, acetic, lactic, citric, malic and tartaric acids in must has been reported by a number of workers,[6-18] but less attention has paid to the effects of fermentation conditions on the formation of these organic acids.

This paper describes the effects of different wine yeast strains and of such environmental conditions as temperature, pH, and the concentrations of sugar and SO_2 in the must on the formation of organic acids during fermentation. It was found that these factors significantly affected the formation of succinic and acetic acids but not lactic and citric acids.

Materials and Methods

Microorganisms Table 1 lists the yeast strains used. Of the 20, 18 are wine yeasts. *Saké* yeast (*Sacch. cerevisiae* RIB 6001) and beer yeast (*Sacch. carsbergensis* BIG 164) were used for comparison. The wine yeasts *Sacch. cerevisiae* IAM 4274 and *Sacch. cerevisiae* RIFY 7 were used to examine the effects of environmental conditions on acid formation. Strains of *Schizosaccharomyces pombe* and *Schiz. malidevorans* were kindly supplied by prof. Dr. S. Goto of Yamanashi University, the other strains were selected from the yeast culture collection of this laboratory.

Vinification Koshu grapes were harvested in 1977 and 1978 from commercial vineyards at Katsunuma, Yamanashi Prefecture. The grapes were destemmed and crushed, and the must was treated with 200 mg/*l* of pectinase (Sclase, Sankyo Co. Ltd.,) in the absence or presence of 50 mg/*l* of SO_2 in the form of sulfurous acid solution, stored at 5°C for 20 hr, then racked. After filtration, the clear must was ameliorated with sucrose to 22 % sugar, and 200-ml portions were autoclaved in 500-ml glass bottles at 120°C for 15 min. Cultures of the yeast strains in this must at 25°C for 48 hr were used as inocula at a level of 5 % (v/v) for the wine fermentations carried

* Studies on Organic Acids in Grape Must and Wine (III).
This work was presented at the Annual Meeting of the Society of Fermentation Technology, Japan held in Osaka, November 8th, 1979.

out under the conditions described later. After fermentation, the wines were racked and filtered through membrane filters (Millipore, pore size 0.45 μm, Nihon Millipore Ltd.,) and the contents of certain organic acids and ethyl alcohol in the filtrates were determined.

Analyses Organic acids were assayed with a carboxylic acid analyzer, model S-603 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan).[19] Ethyl alcohol was measured enzymatically with a descrete analyzer (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan) by the method of Kanbe *et al.*[20,21]

Results

Effects of yeast strains on formation of organic acids First the effects of the yeast strain used on the formation of organic acids was examined. A fermenting must (4.4 *l*) obtained from Koshu grapes (7 kg) harvested in 1977 was divided into Twenty two 200-ml lots, which were pasteurized in 500-ml glass bottles, inoculated with 10-ml of the yeast cultures, and allowed to ferment at 15°C for 35 days until completion of fermentation. Table 1 shows the amounts of organic acids and ethyl alcohol in the finished wines. Succinic, acetic and lactic acids were all produced by the fermentation, the former two in appreciably different amounts depending on the strain, while citric acid was generally decreased by a small amount. Of the 10 strains of the genus *Saccharomyces*,

Table 1. Contents of various organic acids in wines fermented by different yeast strains.

Yeast strains	Succinic acid	Acetic acid	Lactic acid	Citric acid	Tartaric acid	Malic acid	Ethyl alcohol vol. %
	(g/*l*)						
Sacch. cerevisiae IAM 4274	1.32	0.50	0.04	0.20	2.68	1.31	13.15
Sacch. cerevisiae RIFY 7	1.04	0.42	0.05	0.23	2.58	1.57	12.49
Sacch. cerevisiae BIG 161	0.89	0.49	0.05	0.20	2.63	1.21	12.82
Sacch. cerevisiae BIG 42	0.75	0.75	0.06	0.20	2.76	1.54	13.48
Sacch. delbrueckii BIG 172	0.87	0.83	0.07	0.20	2.78	1.00	12.17
Sacch. italicus BIG 9	0.90	0.79	0.07	0.23	2.75	1.01	12.17
Sacch. oviformis IAM 4325	0.89	0.68	0.06	0.23	2.76	1.54	13.48
Sacch. bayanus IFO 0206	0.58	0.50	0.04	0.15	2.63	1.57	13.15
Sacch. fractuum IFO 1089	0.83	0.81	0.06	0.15	2.73	1.34	13.50
Sacch. bailii NISL TK-1	1.61	0.31	0.13	0.26	2.70	1.23	7.23
Schiz. pombe RIFY 1011	0.78	0.30	0.04	0.12	2.68	0.04	13.81
Schiz. pombe RIFY 1013	0.81	0.33	0.05	0.12	2.73	0.03	12.82
Schiz. malidevoarans AWRI 442	0.66	0.18	0.04	0.15	2.82	0.05	11.18
Br. lambicus BIG 185	0.80	0.52	0.05	0.23	3.53	1.87	6.58
C. krusei IAM 4801	0.33	1.07	0.11	0.25	4.00	1.99	1.20
P. membranaefaciens IAM 4076	0.07	0.25	0.01	0.37	4.05	1.86	0.20
H. anomala IAM 4721	0.21	1.64	0.01	1.21	2.95	1.82	0.70
Kl. apiculata IFO 0865	0.30	1.24	0.09	0.19	3.51	1.89	6.58
Sacch. cerevisiae RIB 6001	0.78	0.37	0.05	0.22	2.88	1.99	12.17
Sacch. carlsbergensis BIG 164	0.78	0.49	0.06	0.15	2.86	1.15	11.84
Must before fermentation	0	0	0	0.24	4.11	2.05	0

Koshu grape must was fermented with various yeast strains at 15°C for 35 days. The fermented wines were racked and sterile-filtered.
IAM: Institute of Applied Microbiology, University of Tokyo, Japan.
RIFY: Research Institute of Fermentation, Yamanashi University, Kofu, Japan.
BIG: Botanisches Institut, Geisenheim am Rhein, Germany.
IFO: Institute of Fermentation, Osaka, Japan.
NISL: Noda Industrial Science Laboratory, Noda, Japan.
RIB: National Research Institute of Brewing, Tokyo, Japan.

Sacch. cerevisiae IAM 4274 and *Sacch. bailii* NISL TK-1 formed large quantities of succinic acid, while *Sacch. bailii* NISL TK-1 produced a relatively small amount of ethyl alcohol (7.23 vol. %). Acetic acid was formed in appreciable amount by *C. krusei* IAM 4801, *H. anomala* IAM 4721 and *K. apiculata* IFO 0865 and in relatively small amount by the three strains of the genus *Schizosaccharomyces*. Lactic acid was produced in concentrations of from 0.01 to 0.13 g/*l* (average value 0.05 g/*l*) with *Sacch. bailii* NISL TK-1 and *C. krusei* IAM 4801 producing the largest amounts. *H. anomala* IAM 4721 produced a considerable amount of citric acid (1.21 g/*l*). The amount of tartaric acid remaining in the wines seemed to correlate with the amount of ethyl alcohol formed, except in the cases of *H. anomala* IAM 4721 and *Schiz. malidevorans* AWRI 442. The three strains of *Schizosaccharomyces* metabolized malic acid in the musts almost completely. This result agreed with previous observations.[22-28]

Effects of environmental conditions on formation of organic acids during fermentation The effects of environmental conditions on the formation of organic acids were investigated with *Sacch. cerevisiae* IAM 4274 and *Sacch. cerevisiae* RIFY 7, both of which produced a large amount of succinic acid. Must (7.8 *l*) from Koshu grapes (12 kg) harvested in 1978 was divided into 39 200-ml lots, which were pasteurized in 500-ml glass bottles at 120°C for 15 min. The amounts of tartaric, malic and citric acids in the pasteurized must were respectively 4.20, 1.55 and 0.25 g/*l*.

Effects of fermentation temperature First the effects of fermentation temperature on the formation of orgnaic acids were examined. Cultures of *Sacch. cerevisiae* IAM 4274 and *Sacch. cerevisiae* RIFY 7 incubated in 10-ml of sterile must at 25°C for two days were inoculated into 200-ml of must and fermented at 10 to 40°C for 35 days. The musts were then racked and sterile-filtered, and the amounts of organic acids and ethyl alcohol in the filtrates were determined.

As Fig. 1 shows, succinic acid formation

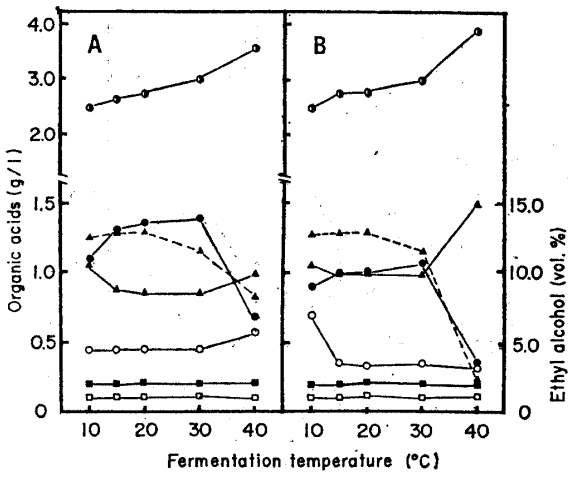

Fig. 1. Effect of fermentation temperature on organic acid contents of wines fermented by *Sacch. cerevisiae* IAM 4274 (A) or *Sacch. cerevisiae* RIFY 7 (B).
Koshu grape must was fermented at 10, 15, 20, 30 or 40°C for 35 days.
Symbols: ●, succinic acid; ○, acetic acid; ■, citric acid; □, lactic acid; ◑, tartaric acid; ▲, malic acid; ▲, ethyl alcohol.

by both strains increased with the elevation of fermentation temperature from 10 to 30°C and decreased remarkably at 40°C. This decrease may be due to the derangement of cell metabolism.[29] There was a noticeable difference in temperature effect on acetic acid formation between *Sacch. cerevisiae* IAM 4274 and *Sacch. cerevisiae* RIFY 7. *Sacch. cerevisiae* IAM 4274 produced most acetic acid at 40°C, while *Sacch. cerevisiae* RIFY 7 produced a large amount (0.71 g/*l*) at 10°C. Slow fermentation may account for the accumulation of acetic acid by *Sacch. cerevisiae* RIFY 7, as has been described by Schanderl.[30] The amount of lactic and citric acids formed at 10 to 40°C were almost constant, approximately 0.1 and 0.2 g/*l*, respectively. Tartaric acid contents increased in parallel with the decrease of ethyl alcohol concentration. On the other hand, the amount of malic acid in the wines was almost constant between 15 and 30°C.

Effects of sugar concentration Filtered must of 15.3% sugar content was ameliorated with sucrose to contain 22 to 35% sugar and autoclaved (120°C, 10 min). The sterilized must (200-ml) was inoculated with yeast cultures pre-incubated under the conditions described in the preceding paragraph. After

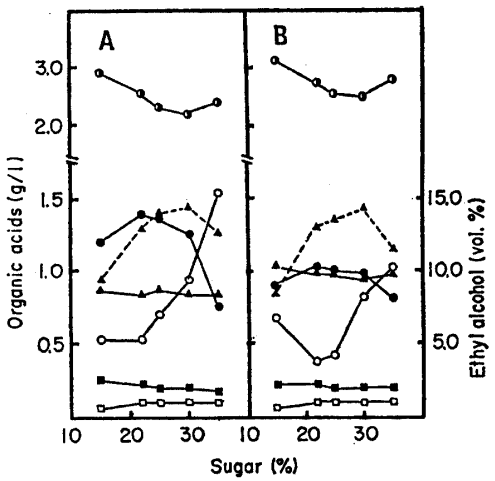

Fig. 2. Effect of sugar concentration in must on organic acid contents of wines fermented by *Sacch. cerevisiae* IAM 4274 (A) or *Sacch. cerevisiae* RIFY 7 (B).
Koshu grape musts of different sugar contents (15, 22, 25, 30 or 35 %) were fermented at 15°C for 35 days.
Symbols: ●, succinic acid; ○, acetic acid; ■, citric acid; □, lactic acid; ◑, tartaric acid; —▲—, malic acid; ---▲---, ethyl alcohol.

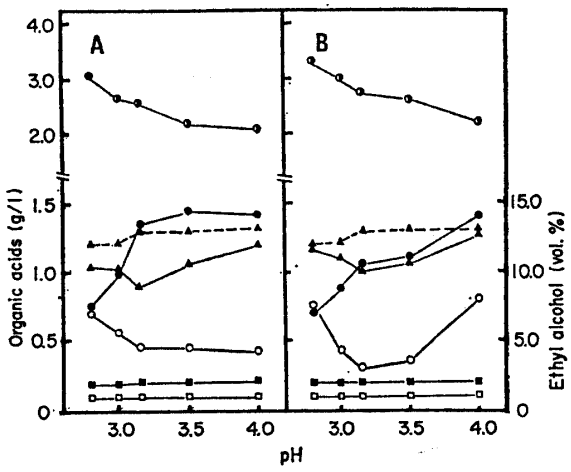

Fig. 3. Effect of pH of must on organic acid contents of wines fermented by *Sacch. cerevisiae* IAM 4274 (A) or *Sacch. cerevisiae* RIFY 7 (B).
Koshu grape must of pH 2.8, 3.0, 3.15, 3.5 or 4.0 was fermented at 15°C for 35 days.
Symbols: ●, succinic acid; ○, acetic acid; ■, citric acid; □, lactic acid; ◑, tartaric acid; —▲—, malic acid; ---▲---, ethyl alcohol.

fermentation at 15°C for 35 days, the amounts of organic acids and ethyl alcohol in the finished wines were determined (Fig. 2). Succinic acid content was highest in musts ameliorated with sucrose to 22% in both cases. Ethyl alcohol was maximum in musts with 30% initial sugar. Acetic acid significantly increased with the concentration of sugar from 22 to 35%. These retults are in good accord with those of Schanderl[80] and Totsuka *et al.*[14] Formation of malic, citric and lactic acids was virtually independent of sugar concentration. An inverse correlation was also found between tartaric acid and ethyl alcohol at each concentration of sugar.

Effects of initial pH of must Must was ameliorated with sucrose to 22% sugar, divided into ten 200-ml portions, and adjusted to 2.8, 3.0, 3.5 and 4.0 with 1 N-HCl or 1 N-NaOH. Original must (pH 3.15) was used as control. The must was pasteurized and fermented at 15°C for 35 days, followed by analysis.

As Fig. 3 shows, succinic acid formation increased with increasing pH of the musts. In the case of acetic acid, *Sacch. cerevisiae* IAM 4274 produced an almost constant amount in the range of pH 3.15 to 4.0, while *Sacch. cerevisiae* RIFY 7 showed remarkable dependence on pH. Namely, the effects of initial pH of the musts on acetic acid formation was different for different yeast strains. No such interstrain difference was found in the formation of lactic and citric acids under these conditions. Less tartaric acid remained in the wines produced from musts of higher initial pH. Malic acid was lowest in both wines fermented at pH 3.15.

Effects of SO_2 concentration in must Must was ameliorated with sucrose to 22% sugar and divided into eight 200-ml portions, which were treated with 0 to 150 mg/*l* of SO_2 in the form of sulfurous acid solution. The must was then fermented with the two strains of yeast at 15°C for 35 days.

Figure 4 shows that production of succinic acid decreased gradually with the increase of SO_2 concentration. *Sacch. cerevisiae* RIFY 7 produced considerably more succinic and acetic acids without SO_2 treatment than with it, and in this respect seems to be more sensitive to SO_2 than strain IAM 4274. The effects of SO_2 on the formation of succinic and acetic acids were similar for both yeast strains. Initial concentration of SO_2 in the

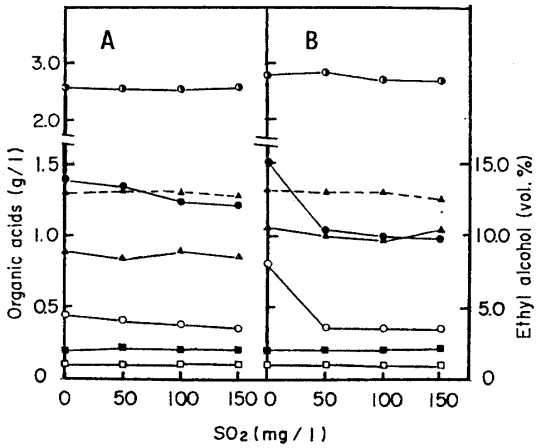

Fig. 4. Effect of SO_2 concentration in must on organic acid contents of wines fermented by *Sacch. cerevisiae* IAM 4274 (A) *Sacch. cerevisiae* RIFY 7 (B).
Must treated with 0, 50, 100 or 150 mg/*l* of SO_2 was fermented at 15°C for 35 days.
Symbols; ●, succinic acid; ○, acetic acid; ■, citric acid; □, lactic acid; ◑, tartaric acid; —▲—, malic acid; ---▲---, ethyl alcohol.

musts had no apparant effect on lactic, citric, tartaric and malic acids in either strains.

Discussion

Significantly different amounts of succinic and acetic acids were formed from musts by wine yeasts under the various environmental conditions. The average amounts of succinic and acetic acids formed by four strains of *Sacch. cerevisiae* in the pasteurized musts were approximately 0.9 and 0.5 g/*l* of wine, respectively. These are remarkably higher than the amounts of succinic acid (0.4 g/*l*) and acetic acid (0.3 g/*l*) formed by *Sacch. cerevisiae* var. *ellipsoideus* IFO 2313 in sterile-filtered musts without pasteurization.[17] This suggrests that *Sacch. cerevisiae* may produce more succinic and acetic acids in pasteurized musts than in sterile-filtered musts. High sugar concentration in musts resulted in considerable formation of acetic acid by *Sacch. cerevisiae* (Fig. 2). This suggests that *Sacch. cerevisiae* may produce a large amount of acetic acid during fermentation of botrytized musts with high sugar concentrations of over 30%.[11] All strains of *Sacch. cerevisiae* produced very small amounts of lactic and citric acids (0.04–0.23 g/*l*), which were not affected by the environmental conditions or the difference in strains. These contents of lactic and citric acids closely matched those of commercial table wines.[22]

In sum, the differences in the strain of wine yeast and the fermentation conditions of the musts were found to influence considerably the formation of succinic and acetic acids. As normal by-products of alcoholic fermentation by yeasts, succinic and acetic acids are known to play a definite role in the organoleptic quality of wines.[8,18] Therefore, proper control of the formation of the organic acids during fermentation must be considered a very important part of wine making.

Acknowledgments

The authors wish to thank prof. Dr. S. Goto of Yamanashi Univ. and Dr. S. Nasuno of Kikkoman Corporation, for their valuable advice. The authors also thank Drs. N. Iguchi and F. Yoshida of Kikkoman Corporation, for their encouragement.

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(Received September 5, 1980)