Effect of pH, Temperature and SO_2 Concentration on the Malo-Lactic Fermentation Abilities of Selected Bacteria and on Wine Colour*

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Thirty malo-lactic bacterial cultures, isolated from red wines from 10 Western Cape wineries, were tested for their malo-lactic fermentation abilities in Cinsaut wine. Each isolate was tested at SO_2 concentrations of 34 and 61 mg/ℓ pH levels of 3,5 and 3,8, and temperatures of 15 and 20 °C. The wines were inoculated after alcoholic fermentation, and malo-lactic fermentation was followed by paper chromatography tests for the following 5 months. At this point the colour of each bottle of wine which had undergone malo-lactic fermentation, was determined and the colour loss calculated.

The lower SO_2 concentration and higher temperature were significantly more favourable to malo-lactic fermentation. Two of the *Leuconostoc oenos* isolates completed malo-lactic fermentation highly significantly more rapidly than nearly all other *L. oenos*, *Lactobacillus* and *Pediodoccus* isolates under the conditions tested. No great differences, few of which were significant, were found amongst the other isolates. The higher SO_2 concentration was the most important single factor causing colour loss in the experimental wines. The 30 bacterial cultures tested did not produce significantly different losses in colour.

Malo-lactic fermentation (MLF) entails the bacterial conversion of malic acid (a dicarboxylic acid) to lactic acid (a monocarboxylic acid) and CO_2, thus partially deacidifying the wine (Suverkrop & Tchelistcheff, 1949; Ingraham, Vaughn & Cooke, 1960; Ribéreau-Gayon & Peynaud, 1964; Rankine, 1970), and occurs naturally in the dry wines of many countries (Kunkee, Ough & Amerine, 1964). In countries with colder climates where the wines sometimes have very high acidities, MLF is an important means of deacidification. In South Africa MLF poses a problem in wines which already have pH-values (Van Wyk, 1976) unless the pH can be satisfactorily decreased by the addition of acid. Malo-lactic fermentation is the most effective means of ensuring that a wine is biologically stable (Ribéreau-Gayon & Peynaud, 1964; Maret & Sozzi, 1977; Rankine, 1977); however, it should not occur after bottling as it may produce an undesirable haze and gas in the wine (Ribéreau-Gayon & Peynaud, 1964; Rankine & Bridson, 1971). The only practical way to ensure a swift MLF is inoculation with a suitable strain of malo-lactic bacteria (Vaughn & Tchelistcheff, 1957; Castino, Usseglio-Tomasset & Gandini, 1975; Lafon-Lafourcade, 1975; Beelman, Gavin & Keen, 1977). In a survey of the incidence of MLF in South African wines, Van Wyk (1976) found that approximately the same percentage of award-winning and non-award-winning red table wines (56,9 and 58,1%, respectively) had undergone MLF, indicating no decisive effect of MLF on wine quality and contradicting the general belief that MLF was detrimental (through deacidification) to the quality of red table wines in warm regions.

One of the most important factors affecting MLF is pH. Malo-lactic fermentation takes place most readily in low-acid wines (Ribéreau-Gayon & Peynaud, 1964; Bousbouras & Kunkee, 1971; Van Wyk, 1976; Rankine, 1977). *Leuconostoc* species are able to decompose malic acid at lower pH-levels than most other bacteria (Rankine, 1970; Castino *et al.*, 1975), and one species may even effect MLF at pH 3,0 where all growth of this species is inhibited (Lafon-Lafourcade, 1975).

Although SO_2 is strongly inhibitory or even lethal to malo-lactic bacteria, it does not by itself completely inhibit MLF (Suverkrop & Tchelistcheff, 1949; Fornachon, 1957; Kunkee, 1967a) at concentrations used in good wines. Malo-lactic fermentation has occurred in wines containing SO_2 at concentrations up to 130–160 mg/ℓ (Suverkrop & Tchelistcheff, 1949; Kunkee, 1967a). Low pH has a synergistic effect on the inhibitory activity of SO_2 (Kunkee, 1967 a; Castino *et al.*, 1975). Sugars, aldehydes and anthocyanin pigments readily bind SO_2 (Vaughn, 1955; Kunkee, 1967b; Rankine, 1977) so that the concentration of free SO_2 in dry red wines is usually less than 4 mg/ℓ (Rankine, 1977).

The bound form of SO_2 is less active than the free form against malo-lactic bacteria (Vaughn, 1955; Kunkee, 1967b; Rankine, 1977), but it may also inhibit MLF (Kunkee, 1967b; Lafon-Lafourcade, 1975). The inhibitory effect of bound SO_2 may be due to bacterial attack on the aldehydes, which would set SO_2 free (Fornachon, 1963). A decrease in pH shifts the equilibrium of the reactions

$$SO_3^{2-} + H^+ \rightleftharpoons HSO_3^- + H^+ \rightleftharpoons SO_2 + H_2O$$

in the direction of molecular SO_2 which as a more potent effect on bacterial growth than the two ionic forms (Carr, Davies & Sparks, 1976). However, MLF is possible at SO_2 concentrations where bacterial growth has been stopped (Lafon-Lafourcade, 1975). Heterofermentative

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cocci appeared to be more sensitive to SO_2 than other malo-lactic bacteria (Fornachon, 1963; Carr, Davies & Sparks, 1976).

It is generally accepted that low temperatures delay MLF while higher temperatures, within limits, accelerate it (Webb & Ingraham, 1960; Tchelistcheff, Peterson & van Gelderen, 1971; Rankine, 1972). Lafon-Lafourcade (1975) found that L-malic acid was degraded more rapidly at 30 °C than at 25 or 20 °C. *Lactobacillus brevis* and *L. oenos* were able to complete MLF even at 11 °C, although *L. brevis* took 68 days to complete the fermentation. Peynaud (1956) found that temperatures above 30 °C slowed down MLF.

It is clear that the various factors mentioned above affect MLF to various extents. Because the effect of these factors on bacterial isolates from South African wines was totally unknown, isolates from these wines were tested for their MLF abilities under different combinations of the different conditions.

MATERIALS AND METHODS

Treatments: Thirty malo-lactic bacterial cultures isolated from red wines from 10 Western Cape wineries were tested for their MLF abilities in Cinsaut wine. Each isolate was tested at SO_2 concentrations of 34 and 61 mg/ℓ, pH levels of 3,5 and 3,8, and temperatures of 15 and 20 °C.

Winemaking procedures: Cinsaut grapes were harvested at 19,7° Balling and total acid content (expressed as g/ℓ tartaric acid) of 7,0 g/ℓ; the total acid was determined by the method of Amerine and Ough (1974). After crushing and destemming, 40% (m/m) of the juice was drawn off and discarded to leave a larger skin/juice ratio for better colour extraction, and the skins and juice divided into 2 batches (A and B). The SO_2 concentration of batch A was brought to 17 mg free SO_2/ℓ and 37 mg total SO_2/ℓ, while that of batch B was brought to 29 mg free SO_2/ℓ and 75 mg total SO_2/ℓ according to direct titration (Amerine & Ough, 1974). The musts were inoculated with an active culture of *Saccharomyces cerevisiae* strain WE14 of the OVRI in must, to a level of 3% (v/v) and fermented on the skins until the first 10 °B was fermented out, pressed and the juice fermented to dryness at 20 °C. After fermentation the SO_2 concentration of batch A was adjusted to 34 mg total SO_2/ℓ and divided into two lots; the pH of one was adjusted to pH 3,53 and that of the other to pH 3,80 using ca 3N H_2SO_4. The SO_2 concentration of batch B was adjusted to 61 mg total SO_2/ℓ. Batch B was divided into two lots of which the pH of one lot was adjusted to pH 3,54 and that of the other lot to 3,83. The different lots of wine were bottled separately after sterile filtration and sparging with CO_2.

Preparation of inoculum and inoculation procedure: Thirty cultures representative of 119 malo-lactic isolates (79 lactobacilli, 27 leuconostocs and 13 pediococci) from 10 Western Cape wineries were investigated (Table 1). Each was cultured in the modified Rogosa liquid medium of Pilone, Kunkee & Webb (1966) and inoculated into the grape juice medium of Kunkee (1974) to a concentration of 20 mℓ/ℓ when good growth was evident. When the grape juice culture reached the stationary growth phase and a sediment was evident at the bottom of the flask, four bottles of each of the four lots of wine were inoculated with this grape juice culture to a concentration of 5 mℓ/ℓ and stoppered with cotton wool. Half of the inoculated bottles and uninoculated controls of each lot were incubated at 20 °C and the other half at 15 °C.

Detection of MLF: Malic acid was determined qualitatively by paper chromatography (Rankine, 1969) weekly from 2,5 weeks to 8,5 weeks and thereafter monthly up to 5 months. Fermentation time was taken as the number of weeks in which malic acid disappeared completely.

Measurement of colour loss associated with MLF: After 5 months, at the termination of the experiment, the absorbance of each bottle of wine which had undergone MLF, as well as the controls was read at 420 nm and 520 nm against distilled water in 0,5 cm path length cuvettes. The two absorbance values of each wine were added and doubled to give total colour density according to Somers & Evans (1974) for a 1 cm cuvette. Loss in colour was the difference between the mean total colour density of the appropriate control and the wine which had undergone MLF.

Statistical analyses: Means of the duplicates for each treatment were used for all the statistical analyses.

To take into account those bacteria which did not complete MLF before termination of the experiment, reciprocals of the number of weeks necessary for completion of MLF were used. Bacteria which did not complete the fermentation, whether they started it or not, were given the value 0,0001 as their reciprocal value. The standard factorial analysis of variance (Snedecor & Cochran, 1974) was applied both to the duration of fermentation and to the colour loss data, using the four factor interaction as error to calculate F-values, as Bartlett's test (Snedecor & Cochran, 1974) showed no evidence of significant interactions between bacterial strain, temperature, SO_2 level and pH.

Means for the 30 individual bacteria over all conditions and replications were calculated and D-values for 1% and 5% levels determined according to Snedecor and Cochran (1974) in the case of duration of fermentation data. For the loss of colour values, D-values were calculated for differences between the treatments low temperature/low SO_2 concentration, low temperature/high SO_2 concentration, high temperature/low SO_2 concentration and high temperature/high SO_2 concentration.

RESULTS

The effects of three main factors, bacteria, temperature and SO_2 level on duration of MLF, were highly significant. There was also significant interaction between temperature and SO_2, but as the F-values of temperature and SO_2 were so high, this could have been due to a scale effect. The low SO_2 concentration (34 mg/ℓ) and the high temperature (20 °C) had the highest mean reciprocal values and were thus significantly more favourable for MLF than the high SO_2 concentration of 61 mg/ℓ and the low temperature of 15 °C, respectively.

TABLE 1
Codes and identity of bacterial isolates tested for malo-lactic fermentation ability in red wine

Area	Culture Code[a]	Identity of isolate
Helderberg-Somerset West	Ab1	Homofermentative *Lactobacillus* sp.
	Ab2	Homofermentative *Lactobacillus* sp.
	Ab3	*L. oenos*
	Ba2	Homofermentative *Lactobacillus* sp.
	Ba3	Heterofermentative *Lactobacillus* sp.
	Bb4	*L. oenos*
Stellenbosch	Ca3	Atypical *L. oenos*
	Cc3	Homofermentative *Lactobacillus* sp.
	Cc4	*L. plantarum*
	Db1	*L. plantarum*
	Db2	*L. oenos*
	Dc1	Heterofermentative *Lactobacillus* sp.
	Ea1	*L. plantarum*
	Ea2	Atypical *L. oenos*
	Ec3	Heterofermentative *Lactobacillus* sp.
	Fa2	*L. plantarum*
	Fa5	Heterofermentative *Lactobacillus* sp.
	Fb7	*P. pentosaceus*
	Fc8	*L. oenos*
	Ga1	Heterofermentative *Lactobacillus* sp.
	Gb6	*P. pentosaceus*
	Gc3	Homofermentative *Lactobacillus* sp.
Groot Drakenstein	Ha1	*L. plantarum*
	Ha2	Homofermentative *Lactobacillus* sp.
	Hb3	Atypical *L. oenos*
Constantia	Ia1	*L. plantarum*
	Ia8	*L. oenos*
	Ic2	Homofermentative *Lactobacillus* sp.
Agter-Paarl	Jc1	Homofermentative *Lactobacillus* sp.
	Jc2	Homofermentative *Lactobacillus* sp.

[a]Capital letters in the culture codes indicate the different wineries.

The mean reciprocal values of number of weeks for completion of MLF by each of the 30 isolates over all conditions are shown in Table 2. The data indicate that isolates Ca3 and Ia8, which did not differ significantly from each other, were highly significantly better than most other isolates tested, and were significantly better than all other isolates tested, with the exception of isolate Db2, from which Ia8 did not differ significantly. Isolate Db2 was highly significantly better than isolates Ga1, Fa2, Db1, Cc4, Jc1, Ia1 and Ba3 and significantly better than Ec3, Gc3, Ba2, Ea1, Fa5 and Jc2. Isolates Ab3 and Bb4 were significantly better than Ba3.

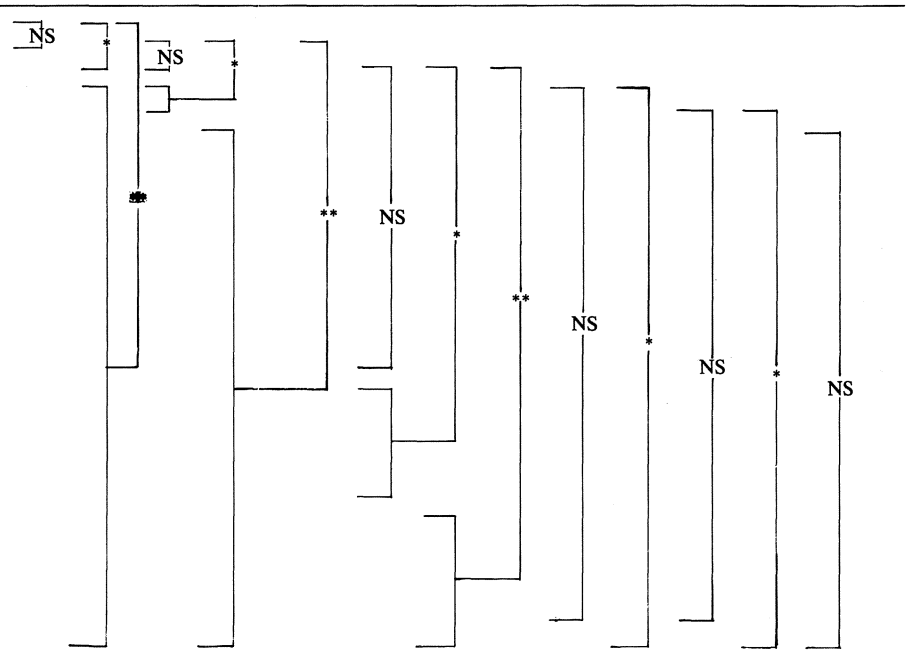
The greatest colour losses were observed with the high SO_2 concentration (61 mg/ℓ). When the reciprocals of colour loss were analysed at the end of the 5-month experimental period, the only significant effects were those of SO_2 concentration (significant at the 1% level) and the interaction between SO_2 concentration and temperature (significant at the 5% level). The colour loss induced by the combination of low temperature and low SO_2 was significantly lower than that obtained with any temperature combination with the high SO_2 concentration (Table 3).

DISCUSSION

In the experimental Cinsaut wine, the lower SO_2 concentration of 34 mgℓ was significantly more favourable for the induction of MLF over the range of 30 isolates than the higher SO_2 concentration of 61 mg/ℓ. This is in accordance with previous findings by others (Vaughn, 1955; Fornachon, 1963; Kunkee, 1967a). The higher

TABLE 2
Significance of difference in duration of malo-lactic fermentation in red wine as affected by 30 different bacterial strains

Isolate[a]	Mean reciprocal weeks	Significance of differences between mean reciprocals[b]
Ca3	0.30	
Ia8	0,27	
Db2	0,17	
Ab3	0,13	
Bb4	0,13	
Fb7	0,12	
Ic7	0,12	
Ab2	0,08	
Cc3	0,08	
Gb6	0,08	
Hb3	0,07	
Ha1	0,07	
Ha2	0,06	
Dc1	0,06	
Ea2	0,06	
Fc8	0,06	
Ab1	0,06	
Ec3	0,05	
Gc3	0,05	
Ba2	0,04	
Ea1	0,03	
Fa5	0,03	
Jc2	0,03	
Ga1	0,03	
Fa2	0,03	
Db1	0,03	
Cc4	0,03	
Jc1	0,03	
Ia1	0,02	
Ba3	0,01	



[a]Isolates arranged in decreasing order of malo-lactic fermentation ability.
[b]NS, difference between means not significant; *, significant difference between means (5% level); **, highly significant difference between means (1% level).

TABLE 3
Significance of differences in colour losses caused by the different SO_2/temperature combinations

Treatment[a]	Mean reciprocal of colour loss[b]	Significance of differences between mean reciprocals[c]
S_LT_L	1,79	NS
S_LT_H	0,72	*
S_HT_H	0,49	NS
S_HT_L	0,30	

[a]S_L, low SO_2 concentration (34 mg/ℓ); S_H, high SO_2 concentration (61 mg/ℓ); T_L, low temperature (15 °C); T_H, high temperature (20 °C).
[b]Least significant differences:
$D_{(5\%)}$ = 1,18168
$D_{(1\%)}$ = 1,47710
[c]NS, difference between means not significant; *, significant difference between means (5% level).

temperature of 20 °C was also significantly more favourable to the induction of MLF than the lower temperature of 15 °C, and is in agreement with the findings of other workers (Webb & Ingraham, 1960; Rankine, 1972; Lafon-Lafourcade, 1975).

Although higher pH values have generally been considered to be more favourable to MLF (Ribéreau-Gayon & Peynaud, 1964; Bousbouras & Kunkee, 1971; van Wyk, 1976; Rankine, 1977), there was no significant difference between the pH values 3,5 and 3,8 for MLF by the 30 isolates tested. This may be because of the inclusion in the group of eight leuconostocs, which are more tolerant to low pH values (Castino *et al.*, 1975) or because the two pH levels fell within the range where all malo-lactic bacteria bring about the MLF relatively easily.

The MLF was completed in the shortest time under all the conditions tested by two of the *L. oenos* isolates. Their MLF abilities were highly significantly better than those of nearly all the other isolates. No greater differences, few of which were significant, were found among the other isolates.

The most important single factor which affected colour loss in the experimental red wines was SO_2 concentration. The 30 bacteria tested did not produce significantly different losses in colour. The combination of the low SO_2 concentration (34 mg/ℓ) with the low temperature 15 °C was not significantly better in respect of the maintenance of colour than the combination of this low SO_2 with the high temperature (20 °C) but was significantly better than both the combinations high SO_2 (61 mg/ℓ) with low temperature and high SO_2 with high temperature. Thus, the high SO_2 concentration was apparently the most important factor promoting loss of colour in the malo-lactic fermented experimental wines.

In practice it has been found that local wine makers are more interested in fast-growing strains of malo-lactic bacteria than in the slower-growing strains. The *Leuconostoc* isolates which are slow-growing are, however, less affected by adverse conditions when present in large numbers and completed MLF more quickly than any of the *Lactobacillus* isolates which are fast-growing.

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