Vitis 7, 22—49 (1968)

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The importance of yeasts in determining the composition and quality of wines

by

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1. Introduction

Although wine making has been a traditional industry for centuries, it is undergoing rapid technological advancement, and the wine maker now has much more control over the wine-making process than was previously available. This control is most apparent in such areas as temperature control during fermentation and maturation, and the clarification and general handling of wines.

However, wine making is basically a microbiological process involving fermentation of grape sugar to ethanol and carbon dioxide by yeast, and it is in this sphere of microbiology that the wine maker has had little control. Although pure yeast cultures have been used in brewing since the turn of the century, following their introduction by Emil Ch. Hansen, they have been very little used in wineries.

There are several reasons for this. The juice of crushed grapes usually contains a wide range of bacteria, moulds and yeasts, in comparison with the boiled and sterile wort of the brewer, and it can undergo a fermentation without further yeast addition. Also, when pure cultures were first introduced into wine making some extravagent claims were made, implying that the strain of yeast was all important and ordinary grapes would yield superlative wine if fermented with a yeast from a famous vineyard. Thus pure culture yeasts fell into disrepute and it is only in relatively recent years that the use of pure yeast starter cultures has come to be considered seriously as a factor which can influence the composition and quality of wines.

In recognition of this, The Australian Wine Research Institute and its predecessor, the section of Oenological Investigations of the Commonwealth Scientific and Industrial Research Organization (C. S. I. R. O), have been engaged *inter alia* on a study of wine yeasts, which has now been carried out, with some interruptions, for 18 years. The immediate purpose of such work has been to examine and select yeasts for use in Australian wineries and to advise on procedures for use, but the more basic purpose has been to study the yeasts from a scientific point of view to gain some insight into the microbiological and biochemical differences between strains and species, and the underlying mechanisms involved.

The purpose of our work has been to study the yeasts which are actually responsible for the winemaking process. Many studies have been made in various parts of the world on yeasts occurring naturally on grapes and in freshly crushed grape juice, but these studies have generally been incomplete because they have not included the rôle, if any, of the yeasts in the fermentation process.

The time is now appropriate to collate the results obtained for the benefit of workers in this field. The results have been obtained from laboratory and pilot-scale fermentations and experimentation in commercial wineries. Over the 18 year period during which the work has been carried out, the use of selected yeast cultures has increased approximately five times in Australian wine making, and compressed and, more recently, dried wine yeast is now commercially available.

Most Australian wines are now fermented by addition of pure cultures of yeasts, and this is one of the factors responsible for the considerable increase in the number of high quality wines made over this period, and the lesser number of wines showing microbiological faults.

2. Taxonomy of wine yeasts and methods of storage

Wine yeasts comprise yeasts which can bring about a complete alcoholic fermentation of grape juice, without deleterious side effects. They comprise essentially strains and species of *Saccharomyces* with possibly some *Schizosaccharomyces* and other genera. No taxonomic distinction is made between wine and brewing yeasts, and the use to which a yeast is put is not necessarily related to its taxonomy.

Historically wine yeasts were referred to as *S. vini* by MEYEr in 1838 after the substrate in which he found them, and this applied also to *S. pomorum* and *S. cerevisiae*. *S. vini* is still used by some taxonomists but is considered by LODDER and KREGER VAN RIJ (1952) as a synonym of *S. cerevisiae*. *S. ellipsoideus* was reduced to a variety of *S. cerevisiae* by STELLING-DEKKER (1931), and its validity as a variety is now in doubt. Miss. SLOOF of the Centraalbureau voor Schimmelcultures, Delft, and Dr. VAN DER WALT of Pretoria both consider it a synonym of *S. cerevisiae* (personal communications) and our comparisons of authentic cultures support this view. KUDRIAVTSEV (1960) introduced fermentation of maltotriose as a biochemical characteristic of *S. cerevisiae* var. *ellipsoideus* for which he retained the name *S. vini* MEYEN, but it would appear from studies at Delft that this characteristic is too variable to be reliable for taxonomy.

The Institute's collection includes at present 72 yeasts of the genus *Saccharomyces* which have been collected by local isolation and by acquisition from overseas collections. The yeasts under study include representatives of the species *S. cerevisiae, S. carlsbergensis, S. oviformis, S. fructuum, S. chevalieri, S. fragilis, S. pastorianus* and *S. veronae*, which have been identified by the methods of LODDER and KREGER VAN RIJ (1952). The yeasts are stored at 4° C on grape juice agar slopes and in 10 per cent aqueous sucrose solution. The slopes are used for working cultures, and stock transfers are made at six-monthly intervals from the sucrose medium, with one or more preliminary passages in dilute grape juice to ensure that the yeast are transferred in an actively growing condition. This method of storage has been found to be satisfactory and failures due to loss of viability have been rare. No instances of alteration of any of the measured characteristics of the yeasts during storage have been observed.

During the course of the work certain yeasts have been studied more intensively than others, either because of their origin or their use in Australian wine making or because of particular metabolic features of special interest. Some details of these yeasts are given below:

S. cerevisiae	No. 134	Australian port yeast, sometimes used in Australian wineries.
S. fructuum	No. 138	Champagne yeast, used in some Australian wineries.
S. cerevisiae	No. 161	Tokay yeast No. 144, Division of Food Technology, University of California, Berkeley.
S. cerevisiae	No. 162	Champagne yeast No. 146 University of California, Berkeley.

S. cerevisiae	No. 170	Agglomerating yeast locally isolated.
S. veronae	No. 173	Isolated locally from fermenting Sauternes containing 700 ppm SO_2.
S. cerevisiae	No. 183	Swiss wine yeast from Lausanne (No. 127, C. C. Morges).
S. species	No. 205	Swiss wine yeast from Lausanne, resistant to cold (No. 162, frigo vin 42).
S. cerevisiae	No. 213	Local isolate, used in some Australian wineries.
S. cerevisiae	No. 228	Local isolate from Tokay grapes.
S. cerevisiae	No. 275	French champagne yeast.
S. chevalieri	No. 317	University of Bordeaux ("Médoc rouge"). Formerly used in some Australian wineries.
S. veronae	No. 318	University of Bordeaux ("St. Emilion rouge").
S. cerevisiae	No. 348	Pasteur Institute, Tunis.
S. cerevisiae	No. 350	Local isolate, used in Australian wineries. Also used for commercial cider making in England and cider research at the Long Ashton Research Station, University of Bristol, as Australian wine yeast 350 R (POLLARD 1966).
S. species	No. 719	Instituto Zimotecnico, Piracicaba, Brazil No. IZ-710.
S. oviformis	No. 723	Local isolate with very high ethanol tolerance.
S. cerevisiae	No. 727	Montrachet strain, University of California, Davis. Used in Californian wineries.
S. cerevisiae	No. 729	Epernay strain used as commercial compressed or dried yeast starter in Australian wineries.
S. carlsbergensis	No. 731	Delft, Holland C. B. S. 1513.

3. Materials and Methods

(a) Laboratory fermentations

These have been carried out in both sterile grape juices and synthetic and defined media of various kinds. The grape juices were prepared from crushed and pressed grapes by sulphiting the juice, settling with pectic enzyme, filtration through coarse filter pads, then through sterilizing pads into sterile glass containers of varying sizes, ranging from 200 ml to 3 l. In early work the juices were heat sterilised, but this tended to give incomplete fermentation, particularly at temperatures above 25° C, and was discarded in favour of filter-sterilised juices. It also prevented quality assessment by tasting. It was found necessary to add a small proportion of an inert sterile solid, such as diatomaceous filter aid or cellulose powder (0.1 per cent by weight), to the bottles to ensure rapid and complete fermentation. This was probably due to oxygen occluded by the solid, which allowed greater yeast growth (see 6, a).

The vessels were closed with cotton wool plugs or lead acetate indicator-tubes to measure hydrogen sulphide evolved, and inoculated with cultures of pure yeasts, in the proportion of 1 per cent by volume of an actively fermenting culture, and allowed to ferment at fixed temperatures within the range 15 to 35° C until fermentation terminated, as determined by loss of weight. The wines were then sterile-

filtered into bottles and stored completely filled in the refrigerator or in a cool room at 15° C until analysed and tasted. With careful technique and prevention of oxidation, he wines made in pure culture in the laboratory compared closely in quality with wines made commercially.

The results summarised in this paper are the results of some thousands of fermentations.

(b) Pilot winery fermentations

The pilot winery of the Australian Wine Research Institute was designed to enable wines to be made in quantities ranging from 1 to 3 hl. The sulphited grape juice was fermented, with or without grape skins and with addition of pure yeast cultures, in 24 stainless steel cylindrical vessels 50 cm diameter and 150 cm deep immersed in thermostatically-controlled water baths. After fermentation the wines were transferred to glass containers of various sizes, stainless steel barrels of 80 l or oak casks of 160 l capacity, and stored at 15° C in a temperature-controlled room. After a period of maturation, the wines were filtered, bottled and stored at the same temperature until tasted and analysed.

(c) Experimental design and statistical analysis

The experiments were designed so that the results could be examined statistically by analysis of variance, and other statistical treatments, such as correlation coefficients and regressions, could be carried out. All fermentations were replicated, including those carried out on pilot-plant scale, and each replicate was treated as a separate fermentation. This experimental design and statistical treatment of the data was regarded as an essential part of the investigations, in view of the variations which can occur in so-called "identical" fermentations. The statistical analyses of the data were generally carried out with a bench calculator, but for the larger analyses a computer was used.

(d) Analytical procedures

The methods of analyses used are briefly as follows:

(1) Sugar content of grape juice as total solids by refractometer or hydrometer.
(2) Ethanol by distillation and immersion refractometer.
(3) pH by glass electrode.
(4) Titratable acidity by electrometric titration with standard alkali to pH 8.4.
(5) l-Malic acid by manometric measurement of carbon dioxide released by conversion to lactic acid using *Lactobacillus arabinosus* (Kolar 1962).
(6) Tartaric acid by the metavanadate procedure or precipitation and titration of potassium bitartrate (Amerine 1965).
(7) Pyruvic acid by enzymatic conversion to lactic acid using lactic acid dehydrogenase (Rankine 1965).
(8) α-Keto glutaric acid by enzymatic conversion to glutamic acid with glutamic acid dehydrogenase using a technique similar to that for pyruvic acid.
(9) Free and bound sulphur dioxide by aspiration into hydrogen peroxide and titration with standard alkali (Rankine 1962).
(10) Higher alcohols by gas chromatography of head-space vapour (Kepner, Maarse and Strating 1964).
(11) Hydrogen sulphide in fermentation gas by measurement of lead sulphide formed in lead acetate indicator tubes (Rankine 1963, 1964). Measurement in wine by the aspiration method of Staudenmayer (1961).

4. Physical aspects of fermentation by wine yeasts

(a) Yeast cell counts

When a yeast culture is inoculated into filtered sterile grape juice and incubated at 20—25° C, haziness first becomes apparent when the total cell numbers reach approximately 100,000 cells per ml, and at 1 million cells per ml the juice is obviously hazy. Fermentation is apparent at 7 to 8 million viable cells per ml and the peak number of viable cells ranges from 50 to 100 million cells per ml (Rankine and Lloyd 1963). This corresponds to a total count of approximately 100 to 200 million cells per ml. For comparison, the initial number of yeast cells in the juice from freshly crushed undamaged grapes ranges from 1,000 to 70,000 cells per ml. The conditions of fermentation influence the cell count considerably, e. g. aeration increases the count, whereas unfavourable media or pH reduces it. In our experience the numbers of yeast cells which develop during fermentation in fresh non-heated grape juice is larger than in any artificial or synthetic medium used.

The most suitable procedures for counting the numbers of yeast cells during fermentation have been found to be the direct microscopic count using a haemocytometer slide for total cell numbers, and an adaption of the microcolony plate count of Miles and Misra (1938) for viable count. The latter involves counting with a low-power stereoscopic microscope the numbers of micro-colonies which grow from viable yeast cells contained in replicated drops of known volume of fermenting medium, which are placed on the surface of malt extract agar in petri dishes and incubated for 16 hours.

(b) Flocculation and clarification

Whilst these characteristics are important in brewing, they are less so in wine making. Yeasts can be graded according to their degree of flocculation, and the deposit after fermentation may be granular, flocculant, powdery or dispersed. Yeasts for champagne-making normally produce a fine sandy deposit and leave the supernatant very clear. The yeasts in our collection have been graded in this fashion and the type of deposit under defined conditions appears to be a characteristic of the strain, and has in some cases been used to check identity in comparative tests. It does not appear to be related to taxonomy.

Sedimentary characteristics of yeasts are of some practical significance since wild yeasts (naturally occurring *Saccharomyces)* frequently remain suspended in the wine after fermentation making clarification more difficult, and the use of a selected strain usually results in more rapid clearing of the young wine. However, with some yeasts very strong flocculation is associated with poor attenuation, in which the yeast flocculates completely before all the sugar in the medium is fermented. This is an undesirable characteristic and is taken into consideration when strains are selected for winery application.

(c) Oxidation-reduction potential

Fresh grape juices before fermentation usually have potentials of 400—450 mV. During fermentation this drops sharply as the oxygen in the medium is removed by the yeast during active growth, and at this stage the medium is well poised and very good agreement is usually obtained between replicate electrodes. At the peak of fermentation the potential reaches its lowest value, usually between 50 and 100 mV, and then rises slowly to some value between 200 and 300 mV. It has been

our practice to record redox potential measurements as millivolts at a stated pH, rather than use the dubious rH term which attempts to combine Eh and pH.

Yeast strain have been found to differ in the lowest potential attained during fermentation, which is a measure of the over-all reducing properties of the yeast, and the time taken to reach this level, which is a measure of rate of growth and fermentation. Measurements made at different depths in fermenting grape juice up to a maximum of 145 cm, in a glass tube 7 cm diameter, showed that the potential was not influenced by depth of fermenting liquid, except for the few centimeters of surface layer which had a somewhat higher potential than the body of the liquid.

The most reliable redox potential measurements have been obtained with replicated platinum electrodes with a square of platinum foil at the tip of the electrode. These have been found to be more reproducable than platinum wire electrodes, but electrodes of either design need to be selected for agreement beforehand. Because of electrode variation, replication of electrodes is essential for accurate readings.

5. Resistance to inhibitors

(a) Sulphur dioxide

Resistance of certain yeasts to sulphur dioxide is well documented and occasionally yeasts with very high tolerance are encountered. We have isolated a strain of *S. chevalieri* from fermenting sweet table wine containing 600 ppm sulphur dioxide, and yeasts with similar or even higher tolerance are sometimes encountered by wine makers. We have also isolated a strain of *Torulopsis bacillaris* from fermenting black-current juice which contained 1200 ppm sulphur dioxide. It is now generally accepted that sulphur dioxide cannot be relied on in all cases to prevent yeast fermentation of sweet table wine, without the wine being rendered unpalatable by the amount added.

Many wines have been examined for yeast spoilage, and occurrence of strains of *Saccharomyces* is common in wines with a low level of free sulphur dioxide. In addition *Saccharomycodes* strains, showing considerable tolerance to sulphur dioxide, have been isolated, and it appears that these yeasts lower the free sulphur dioxide sufficiently by production of acetaldehyde to allow the growth of somewhat lesser tolerant *Saccharomyces*.

Habituation of yeasts to sulphur dioxide by repeated subculture in the presence of increasing doses has been successfully carried out, but is a more difficult process than was envisaged. In our experience it is only after many subcultures, usually more than twenty, that any significant increase in tolerance is acquired. Yeasts have also been habituated to fermentation at elevated temperature and to increased ethanol tolerance.

(b) Other inhibitors

The resistance of yeasts to various inhibitors has been examined in some detail in investigating dominance of pure yeasts added to freshly crushed grape juices containing the natural microflora (Rankine and Lloyd 1963), so that the added yeasts could be identified in the resulting mixed culture. The inhibitors used were actidione, frequentin (an antibiotic produced by *Penicillium frequentans)*, 8-hydroxy quinoline and sorbic acid. These were incorporated separately in various concentrations into replicated malt-extract agar plates (5% malt extract, 2% agar, pH 4.7) which were then inoculated with a range of yeasts and observed at intervals for growth. Many thousands of inoculations were required and a multi-point inoculator similar to that

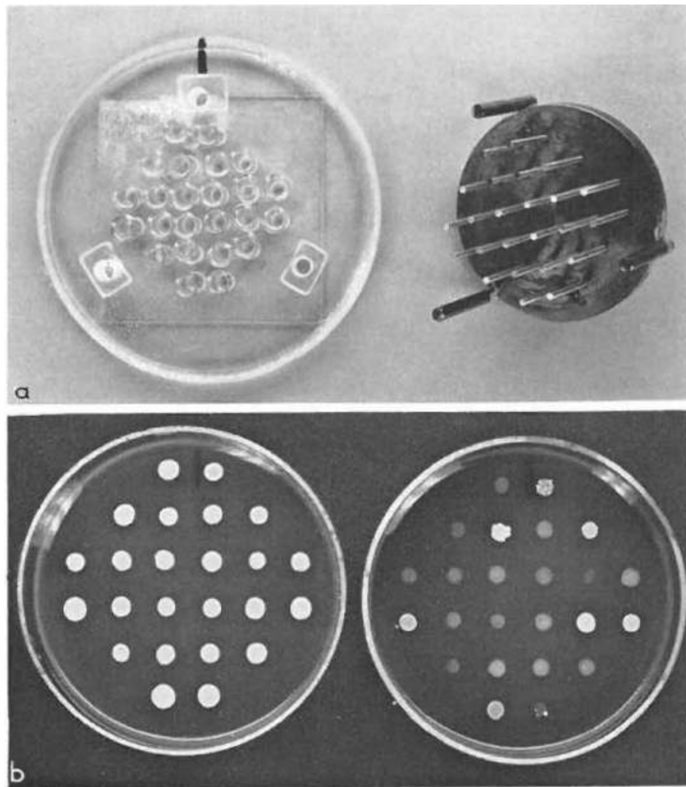


Fig. 1: Multipoint inoculator for simultaneous plating of 24 yeast isolates (a) and examples of growth pattern of 24 yeast isolates in absence (left) and presence (right) of an inhibitor in the medium (b).

described by Beech *et al.* (1955) was used. This multi-point inoculator and the type of results obtained with 24 isolates in the presence and absence of an inhibitor is shown in Fig. 1.

The results of testing 66 *Saccharomyces* strains is shown in Table 1 from which it can be seen that the yeasts differed widely in their resistance to the various inhibitors. When these tests were used in conjunction with giant colony formation, using malt extract plates containing 15% gelatin and 0.1% diphenyl, and sugar fermentation tests, it was possible to determine whether the yeasts isolated from the mixtures were identical with those originally added to each juice. Approximately 70 single-colony isolates were made from each mixed culture studied so that the extent of dominance by the added yeast could be determined statistically by Chi Square tests. The levels of the various inhibitors required were somewhat higher than those found effective in wine (Ribéreau-Gayon and Peynaud 1960) due to the differences in composition between wine and the solid nutrient medium used.

Resistance of yeasts to sorbic acid is topical in view of its occasional use in commercial wine making to prevent the growth of yeasts in sweet table wines. Under these conditions yeasts have been found to vary considerably and strains resistant to 150 ppm sorbic acid (equivalent to 200 ppm potassium sorbate) have been isolated

Table 1

Results of inhibitor tests on 66 *Saccharomyces* yeasts

Inhibitor	Concentration ppm	No. yeasts inhibited
Actidione	0.5	9
	1.0	13
	2.0	46
Frequentin	10	10
	25	60
	50	66
Sorbic acid	100	0
	250	10
	500	65
8-Hydroxy quinoline	75	44
	100	56

from wine. Sorbic acid is thus of less value in wines than was formerly thought, since 150 ppm is near the taste threshold level in wine (Ough 1964) and higher levels impart an off-flavour to the wine, particularly in the presence of bacteria which produce decomposition products.

Tolerance of *Saccharomyces* to diethyl pyrocarbonate (DEPC or PKE) has also been investigated (Rankine 1964). In dilute ethanolic solutions such as wine, this compound has the advantage of having germicidal action without leaving a toxic residue, since it rapidly decomposes to ethanol and carbon dioxide, with a trace of diethyl carbonate. Yeasts were found to differ considerably in their resistance to DEPC when examined in slightly sweet table wine pH 3.5, containing 11 per cent by volume of ethanol and 1 per cent of added glucose. The results are shown in Table 2

Table 2

Influence of D.E.P.C. (PKE) concentration on growth of *Saccharomyces* yeasts in white table wine

(Time in days for visible growth at 25°, means of duplicates)

Yeast	Initial cells/ml	D.E.P.C. (ppm) 0	25	50	100	200
S. cerevisiae No. 170	10^2	12	—*)	—	—	—
	10^4	5	20	26	—	—
	10^6	5	12	26	33	47
S. cerevisiae No. 183	10^2	12	—	—	—	—
	10^4	5	20	—	—	—
	10^6	5	20	26	—	—
S. cerevisiae N. 228	10^2	12	—	—	—	—
	10^4	12	47	—	—	—
	10^6	5	26	26	54	—
S. cerevisiae No. 348	10^2	12	26	—	—	—
	10^4	5	12	33	—	—
	10^6	5	12	20	26	—

*) No growth up to 80 days.

from which it can be seen that strains could tolerate from 25 to 200 ppm under these conditions. The number of yeast cells present at the time of addition of DEPC also influenced the tolerance of the various yeasts, and an increase of 100 fold in cell numbers required approximately double the concentration of DEPC to achieve sterility. Taste threshold measurements were carried out giving a threshold of 600 ppm which agreed with that of KIELHÖFER (1960) and VAN ZYL (1962) but was higher than that found by OUGH and INGRAHAM (1961).

6. Products of fermentation

Fermentation is essentially a microbiological process and the strain of yeast used to conduct the fermentation has been shown to exert considerable influence on amounts of various products of fermentation, which may directly or indirectly be related to wine quality. The results obtained are summarised in the following sections.

(a) Ethanol production

Some yeasts are distinguished from most other microorganisms by their ability to produce a high level of ethanol from sugar. Many yeasts have been examined for this characteristic and wide differences have been observed. An examination of 93 strains of wine yeasts of the genus *Saccharomyces* gave amounts ranging from 7 to 15.5 per cent by volume, the higher value corresponding to complete utilisation of sugar in the grape juice (RANKINE 1953). The results are shown in Fig. 2 which shows the ethanol production of 93 yeasts in pasteurised grape juice containing 23 per cent sugar. From this number, 43 yeasts producing the highest amounts of ethanol were then fermented in grape juice enriched with sucrose to 26.5 per cent sugar and it was found that some yeasts were able to produce more ethanol in the presence of added sugar, whereas other strains could not. A general finding from this and other experiments was that not many strains are able to produce more than 14.5 to 15 per cent by volume in normal fermentation.

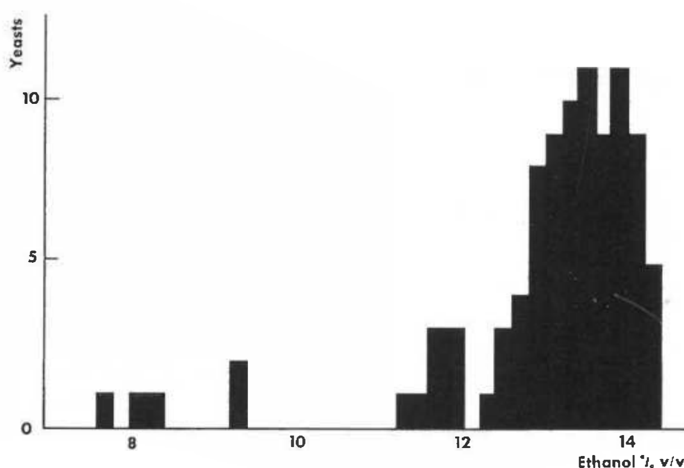


Fig. 2: Ethanol production of 93 strains of *Saccharomyces* in grape juice. Means of quadruplicates.

Table 3

Influence of yeast strain and temperature of fermentation on ethanol production (% v/v) in filter-sterilised fresh grape juice

(Means of duplicates)

Yeast	ethanol	
	16°	30°
S. fructuum No. 138	14.3	12.3
S. cerevisiae No. 134	8.8	13.4
S. cerevisiae No. 275	15.8	12.1
S. cerevisiae No. 278	10.1	12.7
S. cerevisiae No. 350	14.7	13.9
S. oviformis No. 723	16.0	14.3
S. chevalieri No. 317	14.3	12.2
Mean of 24 yeasts including those above	13.7	12.9

L.S.D ($P < 0.05$): 0.7

Temperature of fermentation had an important and a differential effect. Less ethanol was formed generally at higher fermentation temperatures but yeasts differed in their temperature optima. Examples are shown in Table 3, in which the results of 7 yeasts have been selected from a total of 24 tested under comparable conditions. The differences between strains and the interaction of yeast and temperature is clearly shown. High ethanol tolerance has been shown particularly by *Saccharomyces oviformis,* and this has been found by other workers (Ribéreau-Gayon and Peynaud 1960, Scheffer and Mrak 1951, Minarik 1964).

The composition of the medium used for examining ethanol production of yeasts is important. More rapid fermentation occurred in filter-sterilised fresh grape juice than in the same juice after heat sterilisation or in various artificial media of the same sugar concentration, and at higher temperatures of fermentation (30—35° C) the yield of ethanol was higher in the unheated juice. This was partly due to alteration of the composition of the medium by heat and partly to the absence of dissolved oxygen, since faster fermentation and increased cell numbers were obtained by bubbling sterile oxygen through heat-treated medium. An inert porous solid increased the rate of fermentation although there was little difference in the final quantity of carbon dioxide produced. This is shown in Fig. 3. Similar results have been obtained by Crowell and Guymon (1963) who attributed the increase to oxygen occluded by the inert solid.

One yeast (*S. cerevisiae* No. 350) produced a high yield of ethanol at 25°, but tended to leave some residual sugar at 15°. The culture was plated out and single colonies selected and grown as pure cultures which were then examined for ability to ferment well at 15°. This and further selection yielded a strain with somewhat improved fermentation capacity at 15°, and the difference was maintained in subsequent subcultures.

Yeast strains with high ethanol tolerance have been found in both laboratory and commercial wines. The highest level of ethanol in which active yeast growth has been observed was 18 per cent by volume, which occurred in a slightly sweet flor-type sherry undergoing yeast spoilage. This is not the highest value recorded, however. Levels of 18 per cent by volume of ethanol are frequently produced in

Canadian wine-making using syruped fermentation, involving progressive additions of sucrose during fermentation, and up to 20 per cent has been reported (SCHOENFELD 1952).

(b) Production of higher alcohols

Higher alcohols (fusel oils) are always formed during fermentation and are important secondary products in wine. Their formation by different yeasts was

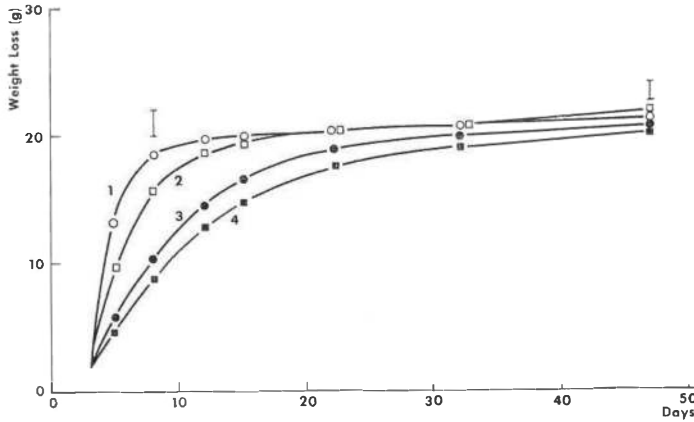


Fig. 3: Influence of pasteurisation of grape juice and addition of inert solid on weight loss during fermentation at 25°.
1. Not heated, diatomaceous filter aid 0.1% w/v added*). 2. Not heated, no addition, 3. Pasteurised, filter aid added, 4. Pasteurised, no addition.

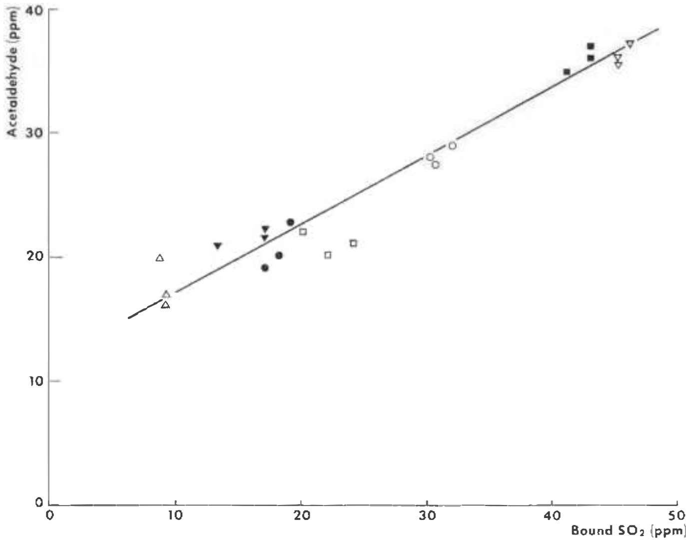


Fig. 4: Relationship between acetaldehyde produced and SO_2 bound (each ppm) by fermentation with 8 *Saccharomyces* yeasts in triplicate in juice from *V. vinifera* var. Ugni Blanc.

*) Both 0.1 and 1.0% inert solid produced similar results.

Table 4

Higher alcohol formation (ppm) by *Saccharomyces* yeasts in three varieties of grape juice.
(Means of duplicates)

Yeast		Variety of grape juice											
		Pedro pH 3.33			Tokay pH 3.34			Ugni Blanc pH 3.42			Mean		
		n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH
S. fructuum	No. 138	20	11	140	107	9	142	52	8	145	60	9	142
S. cerevisiae	No. 213	16	23	95	41	14	126	21	15	125	26	17	115
S. chevalieri	No. 317	12	39	207	26	30	256	10	34	280	16	34	248
S. cerevisiae	No. 350	56	21	151	170	9	151	92	9	164	106	13	155
S. oviformis	No. 723	18	12	111	38	10	128	16	11	146	24	11	128
S. cerevisiae	No. 727	13	41	259	25	38	270	14	31	257	17	37	262
S. cerevisiae	No. 729	13	22	171	24	18	216	18	15	173	18	18	187
S. carlsbergensis	No. 731	9	30	166	19	35	249	11	26	195	13	30	203
Mean		20	25	163	56	20	192	29	19	186	35	21	180

L.S.D ($P < 0.05$)	Means of duplicates	Variety means	Yeast means
n-propanol	22 ppm	12 ppm	24 ppm
iso-butanol	7 ppm	5 ppm	9 ppm
iso- and act.-amyl alcohol	46 ppm	25 ppm	50 ppm

examined (Rankine 1968) by means of gas chromatography which enabled n-propanol, iso-butanol and active and iso-amyl alcohol (combined) to be measured. A range of yeasts of various genera was examined in filter-sterilised juices from three grape varieties. The mean range of values encountered for eight strains of *Saccharomyces* was 115 to 262 ppm for amyl alcohol, 9 to 37 ppm for iso-butanol and 13 to 106 ppm for n-propanol (Table 4). The individual grape juices influenced somewhat the amounts of the various components formed, irrespective of the yeast used. This influence was marginal for amyl alcohol and isobutanol, and of the order of three fold for n-propanol.

Table 5

Characteristics of higher alcohol formation by certain *Saccharomyces* yeasts

Yeast		n-propanol	iso-butanol	amyl alcohols
S. fructuum	No. 138	high	low	—
S. cerevisiae	No. 213	—	—	low
S. chevalieri	No. 317	low	high	high
S. cerevisiae	No. 350	very high	low	—
S. cerevisiae	No. 727	—	high	high
S. carlsbergensis	No. 731	low	high	—

It was possible to select yeasts which produced a high or low level of one or more of the higher alcohols, and examples are given in Table 5, from which it may be seen that the formation of the various higher alcohols is not correlated one with another by the various yeasts, since a particular strain may produce a high level of one alcohol, compared with other yeasts tested, and a low level of another.

The pH and temperature of fermentation influenced the formation of higher alcohols, as shown in Table 6. An increase in the pH of grape juice from 3.0 to 4.2 which is the range of values normally encountered in Australian musts, resulted in an increase in the concentration of all higher alcohols measured. The average increase for four yeasts was 28 per cent for n-propanol, 85 per cent for iso-butanol and 11 per cent for amyl alcohol. An increase in temperature of fermentation from 15^0 to 25^0 resulted in a mean decrease of 17 per cent for n-propanol and an increase of 39 per cent for iso-butanol and 24 per cent for amyl alcohol.

The formation of higher alcohols by certain spoilage yeasts presented an interesting picture. *Schizosaccharomyces malidevorans*, (Rankine and Fornachon 1964) for example, produced an extremely low amount of all higher alcohols although it carried out a complete alcoholic fermentation, whereas *Hansenula anomala*, on the other hand, produced significant amounts of amyl alcohol and iso-butanol when growing as a film, without bringing about any fermentation of the grape juice. This indicated that for these two yeasts the formation of higher alcohols was not related to the fermentation of sugar.

Taste thresholds of the three higher alcohols were measured in dry white table wine with 7 tasters, using triangular taste tests with statistical control, and iso-amyl alcohol was also measured in another dry white wine with strong varietal character as well as in 12 per cent buffered ethanol and in distilled water. The results are shown in Table 7. It can be seen that the taste thresholds for iso-amyl alcohol (the

Table 6

Influence of pH and temperature of fermentation on production of higher alcohols (ppm) by *Saccharomyces* yeasts

(Means of duplicates)

I. pH		3.0			3.4			3.8			4.2		
		n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH	n-PrOH	iso-BuOH	AmOH
S. fructuum	No. 138	56	5	119	51	10	149	62	12	159	51	10	163
S. cerevisiae	No. 213	19	11	106	19	14	108	24	18	136	25	25	162
S. cerevisiae	No. 350	93	9	154	88	8	128	91	11	117	98	15	167
S. cerevisiae	No. 727	18	28	268	18	36	285	18	39	325	21	45	339
Mean		44	13	162	44	17	170	49	20	184	49	24	208

II. Temperature of fermentation		15^0			20^0			25^0		
S. fructuum	No. 138	107	9	142	92	13	175	83	21	192
S. cerevisiae	No. 213	41	14	126	32	21	167	26	22	147
S. cerevisiae	No. 350	170	9	151	157	11	146	156	16	150
S. cerevisiae	No. 727	25	38	270	20	30	323	18	40	362
Mean		86	18	172	75	19	207	71	25	214

L.S.D. ($P < 0.05$):	Means of duplicates		Overall means	
	pH	temperature	pH	temperature
n-propanol	11	8	6	7
iso-butanol	4	6	5	7
iso- and act.-amyl alcohol	34	54	29	27

Table 7

Taste thresholds (ppm) of higher alcohols

(Significance level $P<0.01$)

Taster	Distilled water	iso-amyl alcohol in 12% buffered ethanol	iso-amyl alcohol in Riesling dry white wine	iso-amyl alcohol in Ugni Blanc dry white wine	iso-butanol in Ugni Blanc dry white wine	n-propanol in Ugni Blanc dry white wine
A	3	200	300	200	400	>500
B	5	300	300	300	>500	>500
C	7	700	600	900	>500	—
D	2	100	200	200	500	>500
E	5	600	500	200	>500	>500
F	2	200	300	200	>500	—
G	2	100	100	100	>500	>500
Mean	4	310	330	300	>500	>500

most important component of fusel oil) in the two wines and the buffered ethanol were similar, and approximately 100 times higher than in distilled water. The mean value in wine was approximately 300 ppm, whereas the comparable mean values for iso-butanol and n-propanol were each above 500 ppm. The significance of these results is that differences in amounts of iso-amyl alcohol capable of being formed by *Saccharomyces* can influence the taste of wines, depending on the taster. Differences in the amounts of the other two constituents produced by different yeasts would not affect their taste. This does not take into account synergism between these compounds and the other constituents in wine, which would be very difficult or impossible to measure, and the possible formation of esters. The higher alcohols comprise a portion of the aroma spectrum of wine which is very complex (Drawert and Rapp 1966).

The content of higher alcohols in wines range from 80 to 540 ppm (Guymon and Heitz 1952, Peynaud and Guimberteau 1958, Radler 1960). Our results have shown that the total higher alcohols formed in the one grape juice by a range of yeasts fermenting under comparable conditions can vary from 130 to 310 ppm, indicating that the strain of yeast is an important factor influencing the amounts of higher alcohols in wine. Other factors such as pH of the juice and temperature of fermentation also contribute to this range of values, and Radler (1960) has shown that the year of vintage also has an effect by influencing the composition of the grape juice.

(c) Decomposition of l-malic acid

Although l-malic acid is not a product of fermentation, its concentration in wine is influenced by the yeast strain. l-malic acid and tartaric acid are the two main acids in grape juice, and whereas tartaric acid is biologically stable during fermentation, l-malic acid is partially decomposed. This is distinct from the malo-lactic fermentation carried out by some lactic acid bacteria, in which l-malic acid is decomposed to lactic acid and carbon dioxide.

It was found that decomposition of l-malic acid is widespread among the yeasts examined, comprising 46 yeasts from 5 genera (Rankine 1966). Yeasts from the genus *Saccharomyces* decomposed between 3 and 45 per cent and examples of results obtained are shown in Table 8. The amounts decomposed were greater in highly-acid juice and the pH correlation was statistically significant. The decomposition also

Table 8

Influence of yeast strain and pH on per cent reduction of l-malic acid during fermentation at 25⁰

(Means of duplicates)

Yeast		pH 3.2	pH 3.45	pH 3.72
S. fructuum	No. 138	12	11	8
S. cerevisiae	No. 213	28	23	24
S. chevalieri	No. 317	16	17	8
S. cerevisiae	No. 350	11	10	8
S. oviformis	No. 723	22	16	16
S. cerevisiae	No. 729	25	22	22
S. carlsbergensis	No. 731	30	22	20
Mean		21	17	15

L.S.D ($P < 0.05$) body of table: 5 ppm overall means: 2 ppm

continued when the wines were allowed to stand on their yeast deposit for 6 weeks at 25⁰. In wine of pH 3.2 the amount of l-malic acid decomposed rose from 19 to 24 per cent (mean of 10 yeasts), whereas in wine of pH 3.7 the amount rose from 15 to 32 per cent. The formation of l-malic acid by yeast has been reported in a model system, free from dicarboxylic acids, by DRAWERT, RAPP and ULRICH (1965), but we have not found evidence for l-malate formation during fermentation of grape juice.

Complete decomposition was only observed with *Schizosaccharomyces malidevorans* which produced a stoichiometric yield of ethanol. *Schizosaccharomyces* are known to decompose considerable amounts of l-malic acid (PEYNAUD *et al.* 1964) but as far as is known this is the only yeast which brings about complete decomposition. Unfortunately the growth rate is slow and much hydrogen sulphide is produced during fermentation, which renders its use undesirable in practice. As a matter of interest, not all *Schizosaccharomyces* produce a quantitative yield of ethanol, e. g. DITTRICH (1963) found that ethanol was formed to the extent of 48 and 68 per cent with the two strains he was studying. Two oxidative yeasts tested, a *Pichia* sp. and *Candida mycoderma* consumed 24 and 38 per cent respectively whilst growing as surface films, but the end products of metabolism were not examined.

It is probable that most of the l-malic acid decomposed by *Saccharomyces* is converted to ethanol and carbon dioxide, and although a trace of succinic acid was found, acid-balance studies indicated that l-malic acid is decomposed basically to non-acidic products. The reaction is stereospecific since only l-malic acid was decomposed.

Differences between yeasts in the amount of l-malic acid decomposed suggest a practical application of yeasts in controlling the amount of l-malic acid metabolised during fermentation. By selection of the appropriate yeast strain it is possible to control the extent to which l-malic acid is broken down, and situations exist in which both maximum and minimum decomposition would be desirable, depending on the initial acidity of the grape juice, and such control would be of considerable advantage.

(d) Hydrogen sulphide production

Wines made in our experimental winery and also in commercial wineries occasionally smelt strongly of hydrogen sulphide and no information was available in the literature to adequately explain this phenomenon. Our investigation revealed that the strain of yeast was the basic cause of the aroma, and with proper selection of yeast strain this aroma could be completely prevented. In all, 64 yeasts from 12 genera were examined by fermentation and hydrogen sulphide assay, and strains were found to vary greatly in amounts produced, ranging from no detectable amount to high production readily detectable by smell, even in small laboratory scale experiments (Rankine 1964). The yeast producing the greatest amount was *Schizosaccharomyces malidevorans*, whilst certain *Saccharomyces* strains produced the least, but differences in productivity were not related to taxonomy. Generally about five times as much hydrogen sulphide was produced at 30° as at 15° and about four times as much produced at pH 3.0 as at pH 4.0 (Rankine 1963).

Precursors of hydrogen sulphide were elemental sulphur, bisulphite, thiosulphate, methionine, cysteine and cystine. Maximum production per mole of sulphur was obtained from elemental sulphur, and the amount produced was inversely proportional to the particle size of the elemental sulphur. The addition of sulphate to grape juice did not give rise to hydrogen sulphide during fermentation, but its addition to artificial media resulted in some production. Hydrogen sulphide production from different sulphur compounds by various wine yeasts is shown in Table 9. The amount of hydrogen sulphide produced was measured by the depth of black lead sulphide formed in lead acetate indicator-tubes through which passed the gas evolved during fermentation. This method was quantitative and superceded all other methods examined.

Most hydrogen sulphide was produced at the peak of fermentation when the oxidation-reduction potential was at its lowest level, approximately 80 mV in wine at pH 3.5. After fermentation the yeast deposit formed some hydrogen sulphide from elemental sulphur but not from bisulphite — at the pH of wine, sulphur dioxide is present almost entirely as bisulphite. Hydrogen sulphide can also be produced by contact of wine with metallic iron or steel (not however stainless steel), tin, alu-

Table 9

Hydrogen sulphide formation by wine yeasts in filter-sterilised palomino grape juice containing various sulphur compounds

(mm blackening in H_2S indicator tubes. Means of duplicates)

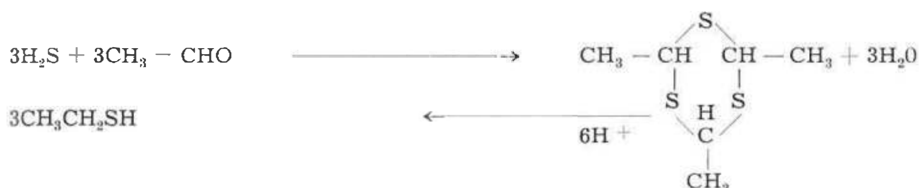
Yeast		No addition	S 10 ppm	SO_2 80 ppm	K_2SO_4 1 g/l
S. fructuum	No. 138	nil	3	6	nil
S. veronae	No. 173	5	10	6	4
S. species	No. 205	tr.	8	7	tr.
S. veronae	No. 318	7	12	7	7
S. cerevisiae	No. 348	nil	5	7	1
S. cerevisiae	No. 350	nil		1	nil
S. oviformis	No. 723	tr.	1	tr.	tr.
Mean		2	6	5	2

L.S.D. ($P<0.05$): 2 ppm

minimum and zinc. This is a direct chemical reduction of bisulphite in the wine by nascent hydrogen developed by reaction between the acids in wine and the metal.

Inhibitor studies indicated that bisulphite reduction did not pass through elemental sulphur, and the presence of hydroxylamine enhanced the formation of hydrogen sulphide from bisulphide, possibly due to its strong reducing properties (RANKINE 1963). Bisulphite is not a common precursor for hydrogen sulphide formation by micro-organisms. Many bacteria produce hydrogen sulphide from cysteine, cystine and thiosulphate but much less from sulphite or bisulphite (CLARK 1953), and d-cysteine disulphurase has been demonstrated in numerous bacterial species. Some *Saccharomyces* yeasts were not able to reduce bisulphite or even elemental sulphur and it is likely that they lack one or both of the enzymes required for sulphite reduction (WAINWRIGHT 1961).

Hydrogen sulphide disappears fairly rapidly in wine particularly after aeration or addition of sulphur dioxide, but if it persists an onion-like aroma develops and ethanethiol has been detected gas-chromatographically in such wines. It is probably formed via the cyclic trithioacetaldehyde intermediate, according to the following reactions (NEUBERG and GRAUER 1952): —



As a consequence of using yeast cultures selected for absence of hydrogen sulphide production the occurrence of wines smelling of hydrogen sulphide has been greatly reduced in both commercial wineries and our own experimental winery. In one case where a selected yeast starter was used and the wine smelled strongly of hydrogen sulphide, the starter culture was found to be contaminated with another yeast which produced a large amount of hydrogen sulphide. In this winery the grape juice used for growing the starter culture was only sulphited and not heat sterilised, and consequently indigenous yeasts resistant to sulphur dioxide were cultivated along with the starter culture, with undesirable results.

(e) Formation of sulphur dioxide — binding compounds

The antioxidant and antiseptic properties of sulphur dioxide reside almost entirely in the free sulphur dioxide moiety in wines, and any compounds which bind sulphur dioxide lower its effectiveness by reducing the level of free sulphur dioxide present. The changes in sulphur dioxide concentration, and distribution between free and bound, have been examined with a range of wine yeasts in grape juices of several varieties, and the results are of considerable interest. In white wines fermented to completion no free sulphur dioxide remains after fermentation and the amount of bound sulphur dioxide remaining is dependent on the concentration of acetaldehyde formed, which in turn is dependent on the strain of yeast used to conduct the fermentation. The amounts of acetaldehyde produced by different yeasts covered a range of up to 4 fold and the close relationship between the amount of acetaldehyde formed and the amount of sulphur dioxide bound during fermentation is shown in Fig. 4.

When sulphur dioxide is added to wine after fermentation it distributes between free and bound and the proportions are largely dependent on the amounts of pyruvic

Table 10

Influence of yeast strain on relationship between SO_2 binding and production of acetaldehyde, pyruvic acid and α-ketoglutaric acid
(Semillon dry white wine. Means of triplicates. All values in ppm)

Yeast	Total SO_2 after fermentation	After subsequent SO_2 addition						calculated SO_2 bound on			sum of calculated bound SO_2	% of measured bound
		free	bound	total	acetaldehyde	pyruvic acid	α-ketoglutaric acid	acetaldehyde	pyruvic acid	α-ketoglutaric acid		
S. fructuum No. 138	6	23	50	73	10	20	14	15	9	3	27	54
S. cerevisiae No. 161	21	39	68	107	19	14	14	28	8	3	39	57
S. cerevisiae No. 275	12	26	74	100	17	61	22	25	7	4	36	49
S. cerevisiae No. 348	17	31	67	98	21	27	16	30	11	3	44	66
S. cerevisiae No. 350	10	19	71	90	22	31	17	32	10	3	45	63
S. cerevisiae No. 719	16	38	64	102	14	26	16	20	11	4	35	55
S. oviformis No. 723	50	36	104	140	37	9	23	54	4	5	63	61
S. cerevisiae No. 729	35	32	82	114	27	23	17	39	9	4	52	63
Mean	21	31	73	103	21	26	17	30	9	4	43	59
L.S.D. ($P < 0.01$):	7	11	12	15	6	7	8					

and α-keto glutaric acid formed during fermentation. The acetaldehyde present is almost completely bound during fermentation and is thus little involved in subsequent equilibria after fermentation.

Table 10 shows the extent of differences which exist between yeasts tested under comparable conditions in relation to the binding of sulphur dioxide. Two other grape juices were examined using the same yeasts under similar conditions, and the calculated amounts of sulphur dioxide bound to acetaldehyde, pyruvic acid and α-ketoglutaric acid for the range of yeasts were 73—83 per cent for juice from Riesling grapes and 61—75 per cent for Ugni Blanc (Syn.: Trebbiano, White Hermitage) juice. The grape juice thus influenced the formation of the three binding compounds as well as did the yeasts. The percentage of bound sulphur dioxide not accounted for is bound to other constituents, which have been investigated by Blouin (1966) for wine and Burroughs and Sparks (1964) for cider. Many compounds are involved but the three compounds examined above appear to be the most important.

Table 11

Influence of pH on formation of pyruvic acid by 5 *accharomyces* yeasts in artificial medium

(Means of duplicates)

Yeast		pH 3.0	3.5	4.0	4.5	5.0	Mean
		pyruvic acid (ppm)					
S. cerevisiae	No. 138	44	94	180	292	352	192
S. cerevisiae	No. 213	90	148	215	283	297	206
S. cerevisiae	No. 350	44	109	180	237	323	178
S. oviformis	No. 723	115	142	253	410	511	286
S. cerevisiae	No. 729	62	112	194	251	278	179
Mean		71	121	204	295	352	208

L.S.D. ($P < 0.05$) body of table: 15 ppm overall means: 51 ppm

Table 12

Pyruvic acid formation (ppm) by wine yeasts in grape juice at 25° C

(Means of duplicates)

Yeast		grape juice			
		Riesling		Doradillo	
		pH 3.18	pH 3.25	pH 3.45	pH 3.72
S. fructuum	No. 138	8	12	47	56
S. cerevisiae	No. 213	16	18	49	98
S. cerevisiae	No. 350	9	10	40	57
S. oviformis	No. 723	17	21	113	120
S. cerevisiae	No. 729	23	19	47	67
S. carlsbergensis	No. 731	14	26	46	53
Mean		15	18	57	75

L.S.D. ($P < 0.05$): 13 ppm.

The formation of pyruvic acid by yeasts has been examined (RANKINE 1965, 1967) and yeast strain and pH appear to be the main factors influencing its concentration in wine. The amount of pyruvic acid formed is strongly correlated with pH of the medium, as shown in Tables 11 and 12. The results in Table 11 were obtained in artificial medium which gave somewhat higher values for pyruvic acid than were obtained in grape juice, but the influence of pH is similar. Various artifical media were investigated and the highest values were found in simple media of low nutritive status, such as buffered glucose with 0.1 per cent Difco yeast extract. Addition of 0.5 per cent peptone reduced the level of pyruvate by approximately 40 per cent, and less still was formed in grape juice which supports good growth of yeasts.

From this and other data an hypothesis has been proposed to account for the influence of pH (RANKINE 1967). It is proposed that pyruvate moves out through the yeast cell membrane by passive diffusion as the undissociated acid, until equilibrium, which is pH dependent, is reached in the external medium or the yeast cell becomes depleted of pyruvate. The finding that the relationship between pyruvate and pH between pH 3 and 4 is logarithmic further supports this hypothesis.

Formation of sulphur dioxide during fermentation (WÜRDIG and SCHLOTTER 1967) occurred with certain yeasts, and examples of the results obtained are shown in Table 13. The ability to form sulphur dioxide appears to be a characteristic of the yeast strain and more is formed at pH 3.6 than 3.0. The source of sulphur dioxide is presumably by reduction of sulphate as the addition of sulphate resulted in more sulphur dioxide formation in juices of the same or similar pH. The result in juice of initial pH 3.6 was influenced by change in pH, due to sulphate addition, which obscured the relative effects of the two variables. Further work is in progress on this subject.

(f) Interrelationship of certain constituents formed or metabolised during fermentation

The interrelation of five of the wine constituents was measured statistically by multiple correlation coefficients. The constituents were ethanol, l-malic acid, pyruvic

Table 14

Interrelationship of certain constituents formed or metabolised during fermentation by *Saccharomyces*

I. Constituents examined

1.	Ethanol	% (v/v)
2.	l-malic acid	g/l
3.	pyruvic acid	ppm
4.	titratable acid	g/l
5.	hydrogen sulphide	mm

II. Correlation coefficients

Constituent		Constituent 2	3	4	5
	1	—0.65***	+0.77***	—0.81***	—0.08
	2		—0.53**	+0.78***	—0.05
	3			—0.61**	+0.10
					—0.02

Table 13

Formation of sulphur dioxide (ppm) by yeast strains during fermentation

(Doradillo grape juice: Sugar 15%, pH 3.4, titratable acid 29 me/l, SO_2 37 ppm. Means of duplicates)

Initial pH	$KHSO_4$ added g/l	*S. fructuum* No. 138			*S. cerevisiae* No. 275			*S. oviformis* No. 723			*S. cerevisiae* No. 729		
		Final SO_2	Change in SO_2	Final pH	Final SO_2	Change in SO_2	Final pH	Final SO_2	Change in SO_2	Final pH	Final SO_2	Change in SO_2	Final pH
3.0	—	10	—27	3.0	28	—9	3.1	44	+7	3.1	31	—6	3.1
3.0	0.56	13	—24	2.9	29	—8	2.9	54	+17	3.0	46	+9	2.9
3.6	—	12	—25	3.7	31	—6	3.6	81	+44	3.7	63	+26	3.7
3.6	0.56	12	—25	3.5	31	—6	3.4	65	+28	3.5	50	+13	3.5
L.S.D. ($P<0.05$):		4			7			25			26		

Table 15

Comparative tests of identity of yeasts isolated from fermenting grape juice against pure yeast culture added before fermentation

Number of isolates tested	Resistance to actidione 0.5, 1.0, 2.0 ppm	Resistance to frequentin*), 10, 20, 30 ppm	Resistance to 8-hydroxy-quinoline, 50, 75, 100 ppm	Appearance of giant colonies	Type of growth in malt extract	Summary
66 from white juice	None differed from starter yeast	2 differed (99, 116) **	1 differed (117)	2 differed (97, 117)	2 differed (97, 117)	4 isolates out of 66 differed from starter, i. e., 6%
69 from red juice	8 differed from starter yeast (3, 9, 45, 60, 64, 65)	4 differed (5, 25, 30, 40)	7 differed (45, 60, 61, 63, 64, 65, 66)	3 differed (45, 64, 65)	3 differed (45, 64, 65)	13 isolated out of 69 different from starter, i. e., 19%

*) An antibiotic produced by *Penicillium frequentans.*

**) Numbers identifying individual isolates showing differences from starter.

acid, titratable acid and hydrogen sulphide and the results are shown in Table 14. With the exception of hydrogen sulphide, all the constituents were correlated significantly ($P < 0.01$). Yeasts which produced a high level of ethanol produced a high level of pyruvic acid and decomposed most l-malic acid. Low l-malic acid decomposition was, naturally, correlated with high titratable acid, since these two measurements related mainly to changes in l-malic acid. Hydrogen sulphide formation was not related to any of the other constituents tested, nor incidentally, to the rate of fermentation or type of flocculation (see 4, b).

The implication from these correlations is that the decomposition of l-malic acid is closely linked to the glycolytic breakdown of sugar via pyruvate to ethanol. The main breakdown products of l-malic acid decomposition appear to be ethanol and carbon dioxide and it is probable that l-malate breakdown couples at some stage with the glycolytic pathway.

The correlations above were all made from results obtained on the same wines, but these did not include measurements of higher alcohols, which were not carried out on these wines. Comparisons were later made, but no relationships were observed between amounts of higher alcohols and the other constituents examined.

Other products of fermentation have been examined, particularly by the Bordeaux School (Genevois 1936, Lafon 1955) and a relationship involving glycerol, succinic and acetic acids, 2 : 3 butylene glycol, acetoin and acetaldehyde, has been established. This relationship has been further investigated by Dourmichidze (1962) with tracer techniques, but the constituents in Table 14 were not examined.

7. Dominance of yeasts in mixed culture

In wine making the fermentation of grape juice is seldom, if ever, carried out by a pure culture, although one strain may dominate the other microflora present. This is because indigenous micro-organisms are normally present on the surface of ripe grapes, and become mixed with the must after crushing the grapes. Moulds are most prevalent, followed by oxidative yeasts and finally fermenting yeasts which are present in very low numbers (Cruess 1918). Consequently, when crushed grapes are allowed to stand in a warm place the juice will normally ferment, although in a few cases we have found that small lots of juices did not ferment but eventually grew mould.

Sulphiting inhibits moulds and many oxidative yeasts, and in a natural fermentation without addition of yeast starter, a succession of microflora usually occurs (Domercq 1957). It has been claimed by Castelli (1955) and others that a succession of micro-organisms influences the quality of the wine, presumably due to different proportions of secondary products produced by oxidative or poorly fermenting yeasts, such as *Kloeckera apiculata* and *Torulopsis bacillaris*. Whilst this may be so we have not investigated such mixed cultures, in the belief that dominance by a selected strain of *Saccharomyces* over all other microflora would be more controllable than endeavouring to establish a manageable succession of yeasts of various genera. The range of yeasts which may be present is demonstrated by the work of Mrak and McClung (1940), Galzy (1956), Domercq (1957) and van der Walt and van Kerken (1963).

Accordingly a series of studies on yeast dominance have been carried out, initially using flocculation characteristics and ethanol yield as measures of dominance (Rankine 1954, 1955), and later by identifying the yeast added before fermentation

and quantitatively assessing the extent of dominance over the naturally occurring microflora (RANKINE and LLOYD 1963).

Studies with mixtures of pairs of *Saccharomyces* differing in ethanol production showed that, with certain strains, dominance as assessed by ethanol production was absolute rather than relative, and depended on the relative proportions of the two yeasts in the inoculum. This implied antagonism between the strains, and was not observed with other yeasts studied (RANKINE 1954).

The influence of mixed cultures on hydrogen sulphide formation has also been investigated (RANKINE 1964). Pairs of yeasts differing in the amount produced have been studied and related to the proportions of the two yeasts at various stages during the fermentation. With a yeast forming a large amount of hydrogen sulphide the amount formed was closely related to the proportion of this yeast in the mixture, but with strains which produced less, the amount was below that corresponding to the proportion of the higher producing yeast in the pair. It appears that when a small amount of hydrogen sulphide is produced the proportion bound chemically in the medium is higher.

Quantitative measurements of dominance of an added pure culture of *Saccharomyces cerevisiae* to freshly crushed grape juice, containing its indigenous microflora, were carried out by sampling the fermentation at different times during its progress. The yeasts in each sample were isolated in pure culture by plating on to grape juice agar and picking off between 60 and 70 isolated single colonies and growing each in sterile grape juice. By the use of various inhibitor tests, which were referred to earlier, these cultures were then compared for identity with the yeast originally added. Examples of results for fermentation of a white and a red juice (the latter with grape skins) are shown in Table 15 from which it can be seen that this type of procedure enabled a quantitative assessment of dominance to be obtained.

From these and other studies the conditions necessary for an added yeast to dominate the fermentation can be defined in general terms. For sulphited and pressed juice from sound freshly picked and crushed grapes, in clean containers free from contact with fermenting must, an inoculum giving a final concentration of 3 million viable cells per ml is desirable. This corresponds to 3 per cent by volume of an actively-fermenting pure yeast starter at the peak of fermentation, which occurs at the stage when approximately half the sugar is consumed.

For red wine fermentation in the presence of grape skins, in which the numbers of indigenous microflora are greater, more inoculum is necessary. The amount required depends particularly on the numbers of indigenous yeasts present and the temperature of fermentation, but 5 million viable cells per ml or 5 per cent by volume of starter is desirable.

8. Influence of yeasts on flavour and aroma

This has been investigated on both pilot winery and large laboratory scale and, in the latter, wines were made by pure yeast cultures in sterile-filtered fresh grape juice in replicated 3 l quantities. The results were of considerable interest and certain *Saccharomyces* yeasts could be identified in masked tastings by the aroma and flavour of the wines produced.

The most important and noticeable flavour was that of hydrogen sulphide, with its associated aroma of ethyl mercaptan (RANKINE 1963). This is characteristics of certain yeast strains which could be readily and regularly identified. Certain other yeasts produced a noticeable quantity of esters, such as ethyl acetate, and fusel oils,

such as iso-amyl alcohol, and wines made by these yeasts could also be identified. The wines made by some other yeasts showed some differences in aroma and flavour which were not reminiscent of pure compounds with which we were familiar. Other yeasts had no influence on the quality of the wines made by them. The yeasts of the genus *Saccharomyces* which produced recognisable flavours and aromas in wines amounted to between 10 and 20 per cent of the yeasts examined. The balance had no recognisable influence on wine quality. The extent of differences in taste due to variations in amounts of higher alcohols was less in wine than that reported in cider (POLLARD, KIESER and BEECH 1966), which suggests that, flavourwise, wine is a more "complex" beverage than cider.

The variety of grape influenced the results obtained by certain yeasts, indicating that an interaction between yeast strain and grape juice occurred. This is not surprising in view of the fact that the composition of grape juice is influenced by the grape variety. The implication is that different grape juices benefit by fermentation with the appropriate yeast strains, but as yet too little quantitative data is available to allow recommendations to be made. In the light of our results of yeast testing over some years it is apparent that proper evaluation of the effect of yeasts on flavour and aroma of wines is technically difficult and requires careful control of experimental conditions. The grape juice must be filter-sterilised and blanketed with carbon dioxide to prevent oxidation, and the resulting wine carefully handled and similarly blanketed. Sterile precautions and sterilised equipment are required. The cold sterilisation of the juice is essential to remove the contaminating influence of other micro-organisms without altering the taste by heating, and sufficient wine needs to be made to allow analysis, maturation and bottling. Differences in composition of wines made by different yeasts can be demonstrated, e. g. VAN ZYL, DE VRIES and ZEEMAN (1963), but it is necessary to determine whether these differences are reflected in changes in flavour of the wines. CROWTHER (1952) has demonstrated flavour differences in wines inoculated with certain film yeasts in Canada, but found less differences with non film-forming yeasts.

Selected yeast cultures are used very little in Europe, for a variety of reasons, some of which are not clear. It is probable that the yeast microflora of vineyards and wineries has become well established over a long period and suitable strains of indigenous *Saccharomyces* exist naturally. However, from our results it would seem that attention paid to microbiological control of fermentation would be well worth while.

Yeast spoilage of wines has been encountered from time to time. We have examined many wines which had unwanted yeast growth causing haziness and, in some cases, gas production. Sweet table wines were most frequently involved and the yeasts isolated were usually strains of *Saccharomyces* which probably carried out the primary fermentation of the grape juice. In many cases the yeast growth was unsightly but did not greatly impair the taste of the wine. However, certain spoilage yeasts may have a profound effect on wine quality.

We have isolated various genera which produce off-flavours in wine and one of these, *Pichia membranaefaciens*, has been reported (RANKINE 1966) because of the unusual circumstances. Wines were made from grapes from the same vineyard in two wineries in an "identical" manner. One wine was normal, whilst the other had a yeasty aldehydic aroma. Both wines contained the normal *Saccharomyces* whereas the spoilt wine also contained *Pichia membranaefaciens*. This yeast was identified and found on inoculation into normal sterile wine to produce the same off-flavour as was present in the wine from which it was isolated. The yeast had an optimum

growth temperature of 20° and could tolerate 11 per cent by volume of ethanol. The products of its metabolism were examined gas-chromatographically and high levels of acetaldehyde, ethyl acetate and iso-amyl acetate were found. Other compounds not yet identified also appeared to be associated with the off-flavour. In subsequent years the winery in question has used a pure yeast starter and no further trouble has been experienced. *Pichia membranaefaciens* is of rather rare occurrence in relation to other spoilage yeasts. Domercq (1956) found only 3 strains in over 2.000 yeasts isolated from Bordeaux musts and did not find it in wine. Castelli (1954) and Minarik, Laho and Navara (1960) likewise found it to be of rare occurrence.

9. Conclusion

From the wine makers' view point at present the advantages of the use of selected yeasts are the rapid and predictable onset of fermentation, its evenness and completion and the absence of undesirable aromas and flavours. However it is now apparent from our work that wine yeasts differ considerably in other characteristics which can also be of oenological importance.

Compounds formed by yeasts which can directly affect the flavour of wines include hydrogen sulphide and mercaptans, iso-amyl alcohol, and ethyl and amyl acetate. Other aroma materials which are not reminiscent of pure compounds, but which affect wine quality, can also be produced. These may confer a quality improvement or, in the case of spoilage yeasts, a reduction in quality. Compounds which indirectly affect the quality of wines are those which bind sulphur dioxide and prevent it from carrying out its antioxidant and germicidal functions. The most important compounds in this connection in normal wines are acetaldehyde, pyruvic and α-ketoglutaric acid, and the amounts produced are influenced by the yeast strain.

It is also possible to exercise control over reduction in acidity during the fermentation by controlling the amount of l-malic acid metabolised by choice of a suitable yeast strain. Likewise some control is possible over the quantities of ethanol and higher alcohols produced and the prevention of off-flavours resulting from fermentation.

One of the most important results of our work has been the prevention of hydrogen sulphide formation in wines. This formation is basically a microbiological reduction of elemental sulphur or sulphur-containing compounds during fermentation, and the control has been to carry out the fermentation with a yeast which is unable to produce hydrogen sulphide. Such yeasts exist naturally and may be selected on the basis of suitable laboratory tests. These yeasts need to have other desirable attributes such as high ethanol tolerance, the ability to carry out a regular and complete fermentation and to be able to grow fast enough to dominate the indigenous microflora.

As a result of our investigations the use of selected yeasts has become widespread in Australian wine making, and the strain of yeast is now regarded as one of the controllable factors necessary to make wines of high and consistent quality.

10. Acknowledgements

It is a pleasure to record my appreciation to J. C. M. Fornachon for his constructive criticism given during the course of these studies. K. M. Cellier of the Division of Mathematical Statistics, C. S. I. R. O., kindly advised on the various statistical treatments of the data.

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Eingegangen am 11. 12. 1967

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