Acetaldehyde Formation in Submerged Cultures of *Saccharomyces beticus*

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The white wines of the Jerez de la Frontera, Sanlúcar de Barrameda, Montilla, and Nova del Rey districts in Spain and of the Château Chalon region in France are produced by a process which differs significantly from that used in all other European districts. In these regions, after the primary alcoholic fermentation the wines are left on all, or a considerable proportion, of the yeast sediment in containers that are not more than 85 per cent full. A film stage of the yeast then develops on the surface of the wine, and, after 1 to several years' growth, imparts a characteristic aldehyde-acetal odor. In many cases, the film remains on the wine for several years bestowing a special nuance of bouquet to the wine. The characteristic film-yeast or "sherry" odor is due not only to the accumulation of acetaldehyde and acetal but also to unidentified substances. Some of these substances appear to be derived from or to follow the autolysis of the yeast. The yeast is a true *Saccharomyces* according to Fornachon (1953). In their fermentation characteristics, these yeasts appear identical to *Saccharomyces cerevisiae* var. *ellipsoideus*, but they are usually classified as *Saccharomyces beticicus* or *Saccharomyces fermentati* (Rankine, 1955 and Castor and Archer 1957). The process has been described in English by Joslyn and Amerine (1941), Cruess (1948), Fornachon (1953a), and others.

Unfortunately, the film process of making a sherry-type wine in casks is slow and expensive. The ratio of surface to volume is most favorable in small containers. Butts of about a 140 gal capacity are employed in Spain. Even so, it takes 3 or more years for the requisite flavor to develop in the wine. Furthermore, the film grows capriciously: its growth is very subject to changes in temperature, and the flavor produced may unaccountably vary from one container to another. This variation in flavor is one reason for the complicated blending system used in Spain in which the contents of many casks are mixed several times during aging. For details of the fractional-blending or solera system employed, see Baker *et al.* (1952).

The production of a film-yeast type wine would be greatly facilitated if the film yeast could be induced to grow in a submerged culture under controlled conditions of sterility, aeration, and temperature, and if the desirable flavor products would be produced and accumulated. This paper reports some experiments which were designed to gain these objectives. The earliest report noted here is that of Fornachon (1953) who made a detailed study of the process of aldehyde formation in shaken suspensions of yeasts in wines. He found that, under aerobic conditions, the cell is able to transfer hydrogen from the reduced coenzyme to molecular oxygen by means of the diaphorase-cytochrome c oxidase system.

Another early report of the submerged culture yeasts of this type with wines is that of Ter-Karapetian (1953). Employing *S. cerevisiae* var. *ellipsoideus* in aerated stirred cultures, he found increases in acetaldehyde and decreases in volatile acidity and total nitrogen. Following aeration, he heated the wine under anaerobic conditions to mature it. The acetal content increased during this period. Ter-Karapetian admitted that the quality of the wines produced was low, and he cautioned that the conditions must be carefully controlled, though his report does not specify precisely what the conditions should be.

Crowther and Truscott (1955) reported that when 2 per cent of freshly cultured film yeast was shaken with wine for 3 weeks, the wine acquired a film-yeast flavor. No chemical data were given, but rapid multiplication of the yeasts was reported, which we have been able to substantiate. Crowther and Truscott (1957) further reported that agitation by pumping over in the presence of flor yeast usually resulted in the formation of a flor character. The agitation was apparently continuous for periods up to several weeks, but agitation of only 3 or 4 min per hr is also stated to be effective. High concentrations of sulfur dioxide were inimical to flor flavor formation. Aldehydes changed in 3 weeks in the 3 wines reported from 65 to 160, 55 to 70, and 95 to 75 mg per L, respectively. These authors found flor character to be associated with accumulation of aldehyde of 100 mg per L or more. In our studies, greater accumulations than this are required if the wine is to have an identifiable "flor" character.

Fornachon (1953a) found a temperature of 68 F (20 C) to be most favorable for the film stage of these yeasts, with less growth at higher and lower tempera-
tures. He also noted that the oxygen content of the air above the film had to be maintained for best film growth, though 80 per cent air and 20 per cent carbon dioxide gave slightly greater aldehyde retention than air alone. Fornachon (1953b), by shaking yeast suspensions in wine, found good aldehyde formation under aerobic conditions, but reported acetic acid to accumulate under anaerobic conditions.

**Materials and Methods**

Filtered grape juice (must) of the 1954, 1955, and 1956 vintages stored at 28 F (−2.2 C) was used for the fermentations. The yeast was no. 519 of the collection of this department. This is a film-forming yeast of the Jerez type, originally acquired from Professor Hugo Schanderl of the Botanische Institut at Geisenheim-am-Rhein. He had isolated it from a Spanish sherry. The fermentations were normal.

The fermentation equipment used was the controlled fermentors previously described by Amerine (1953). These are two 90-L stainless steel fermentors which can be operated at a constant temperature and pressure. About 72 L of must were fermented in each. The fermentors were usually operated at 65, 70, or 80 F, and at times they were operated under pressures of 1 to 8 atm. The gases used were oxygen or carbon dioxide from tanks and air from a compressor. The gas was filtered through a charcoal column before entering the fermentation chamber. The stirrers were stainless steel paddles about 4 in. long. They were operated at 90 rpm, either continuously or automatically a certain number of min per hr. Prior to sampling, the contents were mixed by stirring or pumping over.

In experiments in which alcohol or aldehyde were recovered from gases, about ½ gal of neutral buffer containing sulfite was introduced into the washing column. These solutions were changed and analyzed daily. The analytical procedures were those normally employed in this laboratory as outlined by Amerine (1955). The yeast cell count was made by the direct hemacytometer method, using a Levy-Hausser counting chamber.

**Results and Discussion**

During the experiment with Thompson Seedless must of 1955 vintage in fermentation no. 1, the following sequence of treatments was used after the primary or alcoholic fermentation: the oxygen pressure was maintained at 4 atm the first 10 days by bleeding in about 1 L per minute (Lpm) of oxygen. Thereafter the pressure was brought to 4 atm with oxygen every 2 or 3 days. The pressure loss in 3 days usually brought the pressure down to about 1 atm so that the wine was at all times under a positive oxygen pressure. During the last week, oxygen was run in at atmospheric pressure at the rate of 3 Lpm. The temperature was 65 F (18.3 C) until the last 3 weeks, when it was successively raised to 70, 75, and 80 F (22.2, 23.9, and 26.7 C) at 1-week intervals to

![Figure 1. Fermentation no. 1: Changes in yeast count and acetaldehyde content with Thompson seedless must](http://aem.asm.org/)
stimulate aldehyde accumulation. In this experiment, as shown in figure 1, there was an initial drop in yeast count, then an increase, and finally an increase, at the higher temperatures, a decrease. The acetaldehyde content increased, then decreased, again increased markedly, and finally dropped to a low value. However, the final low value is probably due to removal of the acetaldehyde by the oxygen flow at 80 F. Periodic analyses were made of the various constituents. The data for fermentation no.1 are given in table 1. It shows that malic and lactic acids were among the carbon sources under these conditions. Losses in esters and acetaldehyde (figure 1) may have been due to volatilization. Alcohol was probably lost for both reasons. The result of this experiment was negative as far as aldehyde accumulation was concerned. Yeast growth was stimulated by stirring but decreased at high temperatures.

<table>
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<th>Days</th>
<th>Total Acid</th>
<th>Tartaric Acid</th>
<th>Malic Acid</th>
<th>Lactic Acid</th>
<th>Volatile Acid</th>
<th>pH</th>
<th>Neutral Esters mg Ethyl Acetate /L</th>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
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</tr>
<tr>
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<td>0.22</td>
<td>0.21</td>
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<td>0.04</td>
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<td>3.5</td>
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</table>

Simultaneously with the experiment described above, fermentation no. 2 was operated with another sample of the same lot of Thompson Seedless must (figure 2). The temperature and stirring conditions were the same, but pressure was used for 1 week starting with the 43rd day and, during the rest of the experiment, oxygen at the rate of 0.1 lpm was bubbled through the wine. The changes in acetaldehyde and yeast count were similar to those of fermentation no. 1. The yeast count decreased, increased, and finally decreased at the higher temperatures. The acetaldehyde increased, decreased, increased, and then decreased at the higher temperatures and rates of gas flow. Malic and lactic acids and the esters decreased. The result at the completion of this experiment was negative for aldehyde accumulation. However, the oxygen flow did stimulate yeast growth. When the gas flow was stopped, yeast growth decreased and, when restarted, yeast growth and the aldehyde content increased. High temperatures and high rates of oxygen flow retarded yeast growth and led to losses in aldehyde.

The second 1955 experiment was conducted with a new batch of Tankard and Yeast. At the beginning of fermentation, the yeast was quite active and the acetaldehyde was suppressed. The experiment was carried on with the following conditions: oxygen was supplied at 1 lpm until the 44th day when 1 atm of oxygen pressure was applied. The pressure was increased to 4 atm and this was maintained until the end. The temperature was 65 to 70 F (18.3 to 22.2 C) until the 42nd day when it was 80 F (26.7 C) for the rest of the experiment.
week and it was then reduced to 68 F (20 C). The stirrer was operated 5 min per hr until the 44th day when it was used continuously for 5 days; it was then reduced to 1 min per hr until the end of the experiment. In this experiment, as indicated in figure 3, there was a steady decrease in acetaldehyde. All of the loss from the 20th to the 44th day could be accounted for by the amount recovered in the washing tower. There was a distinct decrease in volatile acidity (0.107 to 0.081 per cent) during the 57 days of the experiment, indicating that acetic acid was a carbon source for the yeast, which is normal for this type of yeast under aerobic conditions. There was also a slight decrease in tartaric and malic acids. The total neutral esters increased. The failure in this experiment was apparently due to lack of pressure in the early part of the experiment and lack of air flow in the last part. Yeast strains that accumulate acetaldehyde did not develop in the fermentor.

With the Aligote wine in fermentation no. 4, aerobic conditions were maintained for the first 28 days, but with only 0.1 lpm of oxygen. The yeast count increased during this period, and aldehydes increased slightly. Starting on the 28th day, about 99 per cent carbon dioxide and 1 per cent oxygen were introduced. This caused a rapid rise and then decrease in yeast count. The aldehyde content dropped but, when that recovered in the washer was noted, there was a slight formation of aldehyde, as shown in figure 4. There was, again, a slight decrease in malic acid and in the volatile acidity and an increase in total neutral esters. In this experiment, some aldehyde accumulation occurred and yeast growth was successful, but the lack of air in the liquid was one factor leading to low aldehyde content from the 45th day. Lack of nutrients for continued yeast growth may have been another factor.

In 1956, grape juice of the 1955 vintage (a mixture of various varieties) was used for the original fermentation (Balling 23.7). Fermentation no. 5 (figure 5) was held at 70 F (22.2 C) and 0.1 lpm of oxygen was introduced throughout the experiment. The alcohol was periodically brought to 14.0 per cent during the test. The aldehyde content increased from 52.5 to more than 200 mg per L. The accumulation was slow and was accompanied by decreasing yeast counts. To stimulate yeast growth, 50 mg per L of ammonia was added on the 17th and 66th days without effect. On the 84th day, 1 mg per L of copper and 5 mg of ferrous iron were added, also without effect. The wine was then fortified to 17 per cent alcohol and stored in wood at 53 F (11.7 C). A careful record was kept of the aldehyde and alcohol caught in the washing trap in this experiment. A total of 61 mg per L of aldehyde was recovered. Total aldehyde accumulation was thus about 300 mg per L. The cumulative alcohol loss amounted to 2.8 per cent. Tartaric acid remained constant during the experiment, but malic acid practically disappeared. Two-thirds of the glycerine was lost and lactic acid more than doubled.

The 1955 must fermented in fermentation no. 6 was the same as that in fermentation no. 3. The gas flow was again 0.1 lpm and the stirrer was operated con-

![Figure 3. Fermentation no. 3: Changes in yeast count and acetaldehyde content with Aligote must](http://aem.asm.org/)
continuously at about 90 rpm. The results are shown in figure 6. Again there was a rise in acetaldehyde, somewhat greater than in fermentation no. 5. In addition, the yeast population fell off less with stirring than without. The same additions of ammonia, copper, and iron were made as in the previous experiment. Surprisingly, the loss in aldehyde and alcohol in the washing tower was slightly less than in the previous experiment. Again the malic acid practically disappeared, lactic acid nearly doubled, and glycerine decreased to one-third its original value. These increases in aldehyde and the characteristic "flor" flavor produced were very encouraging.

On the basis of the 1955 and 1956 experiments, it
appeared that the best conditions for success would be low pressure, intermittent stirring, moderate temperature, and continuous but low rate of introduction of oxygen.

On October 31, 1956, Palomino grapes were harvested, crushed, and pressed and placed in fermentations no. 7 and 8. To each, 7.55 g per gal of calcium sulfate was added and, during the fermentation, 1 lpm of oxygen was bubbled through to accelerate the fermentation. Fermentation no. 7 was at 65 F (18.3 C) and no. 8 at 75 F (23.9 C), but both fermentations were completed in about 9 days. They were then both fortified to 14.5 per cent alcohol, and 1 lpm of oxygen was bubbled through.

In fermentation no. 7, 1 lpm of oxygen was introduced, and the contents were stirred continuously at 90 rpm. The yeast counts and aldehyde contents are shown in figure 7. There was a high initial aldehyde content due to (1) the introduction of oxygen during the fermentation and (2) the lower temperature of fermentation. Some of this aldehyde was lost in the first weeks, but later there was an accumulation of aldehyde. This latter increase appears to have been due to the 100 psig pressure applied from the 25th to the 74th day. Thereafter, the treatment was 15 psig air pressure and 0.1 lpm air. From the 80th to 255th day, 34 to 69 per cent of the contents was removed on 11 occasions. Accumulations of aldehyde to more than 1000 mg per L occurred during this period. The wine added to replenish the content of the fermentation varied in aldehyde content from 18 to 98 mg per L.

The essential features of this experiment which differentiate it from previous studies are the use of high pressure (to induce autolysis) and the continuous operation under about 1 atm of pressure with stirring. The result has been a rapid and continuous production of aldehyde. A temperature of 65 F (18.3 C) has also helped to retain the aldehyde produced. The flavor of the wines was recognizable very easily as being of the flor sherry type.

Fermentation no. 8 was the same as fermentation no. 7 except that the initial fermentation was conducted at 75 F (23.9 C) instead of 65 F (18.3 C). The high gas rates with no pressure led to initial losses in aldehyde. Pressure and slower gas flow gave markedly increased aldehyde. Following dilution (on the 48th day) there was little accumulation of aldehyde until the pressure was released and stirring was started. The yeast count and aldehyde content then both increased sharply. The results are shown in figure 8.

In February, 1957, stored grape juice of the 1956 vintage was fermented in fermentation no. 9. The fermentation was made at 65 F (18.3 C) with flor yeast in 17 days. It was then fortified to 14.5 per cent and kept at 15 psig with an air flow of approximately 0.1 lpm. The data are given in figure 9. The rate of accu-
accumulation of aldehyde was slower than in the previous experiment but was continuous from the 29th day. The addition of 40 μ per L of calcium pantothenate on the 82nd day did not stimulate yeast growth or aldehyde accumulation. The initial increase and decrease are possibly due to an induction phase and have been noted in other experiments.

Fermentation no. 7 was operated from the 235th

Figure 7. Fermentation no. 7: Changes in yeast count and acetaldehyde content with Palomino must

Figure 8. Fermentation no. 8: Changes in yeast count and acetaldehyde content with Palomino must
day in a continuous manner, $\frac{1}{10}$ of the contents being removed each day, and the wine in the fermentor brought back to original volume with white dry wine. The yeast counts and aldehyde content were taken daily. After several days an equilibrium was established. The results are shown in figure 10. The rate of aldehyde production on a continuous basis is higher for a more prolonged period than that on a batch basis. This may be due to the continuous supply of nutrients allowing a continuous multiplication of yeast.
cells while also providing sufficient nutrients for the yeast metabolism to more rapidly produce aldehydes.

The products of most of these experiments were fortified to 17.5 per cent alcohol and placed in 10-gal oak barrels. Taste tests uniformly identified the wines as very characteristic of "flor" sherry wines. The high aldehyde contents imparted to the wines a very characteristic flavor and odor. Whether or not such wines would find consumer acceptance is not known. Relatively low-alcohol wines (15 to 17 per cent) of this type have received significant consumer acceptance in Spain.

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SUMMARY

Experiments are reported on the submerged culture of a flor-type yeast (Saccharomyces beticus or Saccharomyces fermentati). Various combinations of temperature, rate of gas flow, pressure, and stirring were used to induce yeast growth and aldehyde accumulation. Aldehyde contents of up to 1000 mg per L occurred.

Acetic and malic acids, glycerine, and alcohol are carbon sources for the growth of the yeast. The addition of ammonia, copper, and iron did not materially aid yeast growth.

The most satisfactory combination of environmental factors was application of 100 psig pressure with low oxygen flow (0.1 L per min) after the initial fermentation. Reduction of the pressure to 15 psig with the same rate of flow of gas but using air instead of oxygen and intermittent stirring of the contents (25 sec per hr) gave excellent yeast growth and aldehyde formation. Under these conditions, about 50 per cent of the contents could be removed every 2 weeks and the aldehyde content of the product maintained at over 500 mg per L.

This process was more successful when conducted on a semicontinuous basis. By removing \( \frac{1}{10} \) of the contents daily, the aldehyde build-up over the period of the experiment was at least twice as fast as in the batch process.

REFERENCES

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