The Growth of Yeasts in Grape Juice Stored at Low Temperatures[1]

III. Quantitative Studies on Growth of Natural Mixed Inocula

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Grape juice cannot be bottled for retail trade without previous storage to precipitate crude argols. Freshly pressed Concord grape juice is now pasteurized, cooled to 0 C (32 F) and pumped into tanks in rooms held at temperatures from −5.5 to −2.2 C (22 to 28 F) to hold the juice until it is ready to be reprocessed for retail. Frequently yeasts develop in the juice during the storage period.

The yeasts isolated from such stored grape juice have been shown to be strains of the genera *Candida, Torulopsis, Hanseniaspora,* and *Saccharomyces* (Lawrence *et al.*, 1959). It has been shown also that each of these in pure culture is capable of developing relatively high populations in grape juice at 0 C, and that the nonfermentative types, chiefly strains of the genus *Candida,* are true psychrophiles.

It is the purpose of this paper to describe, in quantitative terms, the growth of natural inocula of yeasts in grape juice at 1 C and to recommend methods for estimating from yeast counts the length of time the juice may be held in storage under commercial conditions.

Materials and Methods

One-gallon lots of pasteurized and cooled juice were obtained from cooling lines or directly from storage tanks of commercial grape juice plants. These were transferred carefully to sterilized gallon bottles, using precautions to avoid contamination. A thin layer of sterile mineral oil was poured over the surface of the juice. The mineral oil inhibited mold growth but did not materially affect yeast growth. The bottles of juice were then stored in an ice bath placed in a 1 to 2 C room. Samples were removed periodically for determination of numbers of yeasts. The juices were plated in triplicate at several dilutions, using the medium described previously (Lawrence *et al.*, 1959). Plates were incubated at temperatures of 32, 18, and 1 C. Colonies on the plates of two dilutions were counted after 3 days, 10 days, and 4 weeks, respectively. Some of the colonies of yeasts that developed at each incubation temperature were examined microscopically and many were isolated and identified using the procedures previously described.

Fifty-eight 1-gallon lots of juice were obtained and studied during the pressing seasons of 1954, 1955, and 1956.

Results and Discussion

The 58 one-gallon lots of juices could be divided into 6 general groups based upon the incubation temperatures at which the yeast present in the juices developed colonies. Thus 14 samples (group 1) yielded no counts even when held as long as 11 months; 6 samples (group 2) yielded counts at 1 C; 19 samples (group 3) yielded counts at 1 and 18 C; 12 samples (group 4) yielded counts at 1, 18, and 32 C; 5 samples (group 5) yielded variable counts highest initially at 32 C; and 2 samples (group 6) also yielded variable counts at the 3 temperatures.

Group 1. In 14 of the juices, no growth was obtained for periods of from 5 to 11 months. Six of these 14 juices had been obtained from or near the outlet of enclosed commercial coolers following pasteurization and cooling. Four were obtained from near the bottom of surface coolers before the juice entered the trough. The other 4 were taken from tanks shortly after filling.

Group 2. Six of the 58 samples showed a pattern of yeast development similar to that in table 1. It can be seen that the highest counts were obtained from plates incubated at 1 C, much lower counts from plates at 18 C, and no counts from plates incubated at 32 C. A high percentage of colonies which developed at 1 C were

TABLE 1

Typical series of yeast counts obtained at 32, 18, and 1 C from 1 of 6 grape juices in group 2 stored at 1 C

Storage Time Months	Counts of Plates Incubated at:		
	32 C	18 C	1 C
0	0	0	0
1	0	0	0
2	0	0	0
3½	0	0	0
4½	0	10	12,500
6½	0	1,050	19,500
8	0	0	20,900
9	0	0	9,500

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identified as strains of the genus *Candida*. Three of the 6 juices which showed this pattern of growth had been obtained from surface coolers, 1 from an enclosed cooler and 2 directly from the storage tanks. One of the latter was being filled at the time the juice was obtained. With one exception, all of these juices showed no growth until 4 to 7 months after sampling. The one exception was juice obtained from a tank several days after filling. The growth pattern was similar to those of the other five only in that the colonies developed best at 1 C. The juice yielded a count at 1 C when plated the first time, and the yeasts increased after 1 month of storage. In this table as well as in similar data obtained from other samples, inconsistency in counts is noted not only for different incubation temperatures but also from month to month at one temperature. These variations may be due in part to effects of one species upon another, but the causes appear to be far more complex and as yet unexplained.

It is apparent that, among the 20 juices in groups 1 and 2, 19 juices showed no count for 4 to 5 months and some for 11 months or more. Fourteen of these were obtained from the coolers before contact with tanks or exposure to air contamination and the other 6 were obtained from tanks during or shortly after filling. This is considered to be evidence for the adequacy of the pasteurization process used. The eventual high counts of many of these samples resulted from contamination of these gallon samples during the frequent sampling. The last 10 samples were obtained in 1956 in duplicate, one being used for sampling while the second was held until yeast was obtained from the first bottle. Among 6 samples that yielded counts or fermented after a period of time, 3 of the duplicate bottles yielded no counts and the other 3 yielded considerably lower counts than obtained from the first bottle.

Group 3. Still another developmental pattern (table 2) was shown by 19 of 58 one-gallon lots of juice. It can be seen that no counts were obtained by incubation of plates at 32 C, whereas counts at 18 and 1 C increased with time of storage. These yeasts were mostly nonfermentative, mycelial yeasts, presumably strains of the genus *Candida*. Thirteen of these juices had been obtained directly from storage tanks, 4 from pipelines between enclosed coolers and tank, and 2 from surface coolers. None of the 6 juices from the pipelines or coolers yielded a count when first plated. Before counts were obtained, 2 to 7 months of storage had elapsed. If the juices were contaminated when placed in jugs, the contamination was very low. On the other hand, 10 of the 13 juices from tanks contained viable yeasts immediately after sampling. Of the other 3, 2 were samples from newly filled tanks and the third was from a new tank. The data on these 19 lots of juice are evidence of contamination from the tank and air in the room.

Group 4. Twelve of the 58 juices yielded counts in platings made immediately after the juice was obtained. Subsequent platings showed an increase of yeasts able

TABLE 2

Typical series of yeast counts obtained at 32, 18, and 1 C from 1 of 19 grape juices in group 3 stored at 1 C

Storage Time Months	Counts from Plates Incubated at:		
	32 C	18 C	1 C
0	0	60	0
1	0	61,000	75,000
2	0	91,000	105,000
4	0	17,000	19,000

TABLE 3

Typical series of yeast counts obtained at 32, 18, and 1 C from 1 of 12 grape juices in group 4 stored at 1 C

Storage Time Months	Counts from Plates Incubated at:		
	32 C	18 C	1 C
0	0	0	0
1	490	1,000	410
2	32,000	48,000	12,000
4	195,000	30,000	860

TABLE 4

Typical series of yeast counts obtained at 32, 18, and 1 C from 1 of 5 grape juices in group 5 stored at 1 C

Storage Time Months	Counts from Plates Incubated at:		
	32 C	18 C	1 C
0	1,330	60	0
1	640	310	0
2	240	0	0
4	10	150	0
5	0	0	0
7	20	350	42,350
8	0	500	8,000

TABLE 5

*Relative populations of yeasts from a tank of grape juice when juice samples were obtained at intervals during storage, plated in replicate, and incubated at 32, 18, and 1 C**

Storage Time Months	Yeast Counts from Plates Incubated at:					
	32 C		18 C		1 C	
	Count	Type	Count	Type	Count	Type
0	10	—	60	s, nf	30	—
1	0	—	940	nf	1,700	nf
2	0	—	1,940	—	3,200	nf
3	0	—	3,710	nf	4,200	sf, nf
4	0	—	165,000	sf, nf	169,000	sf, nf
5	80	f	370,000	sf, nf	303,000	nf
6	1,800	f	180,000	f, nf	2,350,000	nf

* f = fast fermenter (*Saccharomyces*); sf = slow fermenter (*Torulopsis*); nf = nonfermenter (*Candida*).

to grow at 32 C, as well as at 18 and 1 C (table 3). These juices often exhibited gaseous fermentation. Colonies identified were fermentative types of genera other than *Candida*. Eight of these juices were obtained directly from tanks and 4 from coolers after pasteurization of the juices. Two of the latter were from surface coolers and 2 from enclosed coolers. The first counts from these latter 2 juices were low and the yeasts did not show marked development until the 4 month plating. This group of 12 juices again present evidence for contamination from the air and from the tanks.

Group 5. A fifth pattern of yeast development is shown in table 4. In each of the 5 juices, the first platings yielded counts highest at 32 C, intermediate at 18 C, and lowest at 1 C. However, the 32 C counts decreased during the early period of storage. In some cases counts increased again in later stages. The 18 C counts were variable, but eventually increased, whereas the 1 C counts always increased during storage. Three of these 5 juices were obtained from tanks, 1 from a surface cooler, and 1 from an enclosed cooler in which line contamination was later demonstrated.

Group 6. The final two juices, one from a surface cooler and one from a tank, showed a growth pattern unlike all the others. Both yielded counts at 32 C but none at 18 or 1 C when first plated and both showed slowly increasing counts of yeasts able to grow at 32 and 18 C. One never yielded a count at 1 C, whereas the other yielded a low count at 1 C after 4 months' storage.

In addition to the study of these gallon lots of juice, the pattern of development of yeasts was studied in juice stored commercially in tanks (Pederson *et al.*, 1959). Samples of juice were removed aseptically from the tanks at intervals during the storage period and plated in the manner described. In general, the data showed that those yeasts that were able to grow at 1 C persisted or increased in the juice during prolonged storage. These yeasts were often nonfermentative, produced abundant mycelia and thus appeared to be species of the genus *Candida* (table 5).

From further study of the data on the relative population of fast fermenters, slow fermenters, and nonfermenters in commercial tanks of grape juice, it was found that a high percentage of yeasts isolated after prolonged storage were nonfermentative types and that these were able to grow at 18 or 1 C but not at 32 C. For example, Pederson *et al.* (1959) found that in series 44, 14 juice samples incubated at 32 C yielded counts from 0 to 2500 per ml and at 18 C from 0 to 20,000. In contrast, on plates incubated at 1 C, 7 samples yielded counts of at least 400,000 and 6 others from 4200 to 48,000. The nonfermentative yeasts of the genus *Candida* were by far the predominating type. In some series, counts on individual samples were as high at 32 C as at 18 or 1 C. Such colonies were usually the smooth, mucoid, and fermentative types associated with the genus *Saccharomyces*. Conversely, the relatively few rapidly fermenting yeasts were obtained at 32 or 18, but not at 1 C.

Discussion

From a general consideration of the data, three facts seem to be well established. The first of these is that the pasteurization process is entirely adequate. Samples of juice obtained directly from the outlets of enclosed coolers or from lines properly laid out and properly cleaned remained in good condition for from 4 to 11 months.

Secondly, pasteurized grape juice is readily contaminated with yeasts when it is exposed to the air in the processing plants and to the storage tanks. Of the 37 lots of juices in which yeast growth developed within 4 months, 26 had been obtained from storage tanks, 6 from troughs of surface coolers, 3 from enclosed coolers, and 2 from contaminated lines. In contrast, of the 21 juices that kept for 5 months or longer, 9 were taken directly from enclosed coolers, 7 from surface coolers before juice came in contact with the trough, and 5 from tanks during or immediately after filling.

The third point is that yeasts found in high concentration in juices showing little evidence of fermentation after prolonged storage are predominantly species of the genus *Candida*. These yeasts are nonfermenting, that is, they do not produce carbon dioxide and alcohol from sugars. Even tanks showing relatively high populations of these yeasts are unlikely to show typical evidence of fermentation of the juice.

In general, among those juices which contained yeasts able to grow at 32 C as well as at 18 and 1 C, gaseous fermentation ensued. It appeared that these yeasts were mostly members of the genus *Saccharomyces*. Tanks of juice in which an increasing count of this type of yeast is noticed should be carefully watched, because the ensuing gaseous fermentation may result in an economic loss.

The variation in the temperature ranges for growth and in the optimum temperature for growth of the isolated yeasts (Lawrence *et al.*, 1959), coupled with the data reported here, partially explains the inconsistency of results of yeast counts obtained under commercial conditions by those workers making routine bacteriological examinations of grape juice. Settling of yeast cells, variations throughout tanks, oxygen relationships, and death of cells are also partially responsible for inconsistencies. On numerous occasions, tanks of juice which had previously shown high counts would later show low counts, and vice versa. Such data were therefore extremely difficult to interpret. The samples obtained in routine commercial analyses were usually incubated at room temperature for short periods of time. Consequently, strains which failed to grow at room temperature, such as the *Candida* sp. described

above, would not be counted. In the few cases in which low temperatures were used for incubation, the plates were incubated for only a few days, which is not long enough to allow cells of the low temperature strains to form colonies. From the data reported here and in a previous paper (Lawrence *et al.*, 1959) it would seem that routine incubation of duplicate plates at 10 C and at room temperature (25 C) would give reliable yeast counts in a sufficiently short time to be valuable to the industry.

At the time when samples of juice are to be plated at 10 and 25 C for counting, replicate dilutions should also be inoculated into fermentation tubes and incubated at the same temperatures. Data so obtained would give plant operators an indication of the numbers and types of yeast present in the juice. If an appreciable count is obtained at 25 C, together with gaseous fermentation of sugar, the operator could expect development of fermentative yeasts in the stored juice. On the other hand, samples that yielded low counts and no evidence of fermentation in tubes at 25 C, even when relatively higher counts of nonfermentative yeasts appear at 10 C, should be an indication that the stored juice is not likely to ferment in the immediate future.

Summary

Quantitative plate counts at 1, 18, and 32 C of 58 one-gallon lots of grape juice held in storage at 1 C indicate that grape juice pasteurized and stored without being subjected to recontamination may be kept for reasonable periods with no development of yeast.

Analysis of results indicates that contamination of juices results from contact with the tanks and other equipment and from air in the tank room.

Differences in types of yeast developing in the juices from various sources are reflected in differences in colony counts from plates incubated at 1, 18, and 32 C.

In some cases, slow growing, psychrophilic, nonfermentative yeasts develop in juice. Occasionally, mesophilic fermentative yeasts develop in juice and result in gaseous fermentation in a few months.

Recommendations are made for predicting the keeping quality of the stored juice on the basis of plate counts and fermentation tests of the yeasts present in the juice.

References

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